



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 38/17, C07K 14/00, C12P 21/02</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/22787</b> <b>(43) International Publication Date:</b> 1 August 1996 (01.08.96)															
<b>(21) International Application Number:</b> PCT/US96/00996 <b>(22) International Filing Date:</b> 24 January 1996 (24.01.96)  <b>(30) Priority Data:</b> <table border="0"><tr><td>08/377,309</td><td>24 January 1995 (24.01.95)</td><td>US</td></tr><tr><td>08/377,311</td><td>24 January 1995 (24.01.95)</td><td>US</td></tr><tr><td>08/377,316</td><td>24 January 1995 (24.01.95)</td><td>US</td></tr><tr><td>08/377,317</td><td>24 January 1995 (24.01.95)</td><td>US</td></tr><tr><td>08/505,012</td><td>21 July 1995 (21.07.95)</td><td>US</td></tr></table> <b>(71)(72) Applicant and Inventor:</b> MURGITA, Robert, A. [US/CA]; 17 Parkside Place, Montreal, H3H 1A7 (CA).  <b>(74) Agent:</b> CLARK, Paul, T.; Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804 (US).		08/377,309	24 January 1995 (24.01.95)	US	08/377,311	24 January 1995 (24.01.95)	US	08/377,316	24 January 1995 (24.01.95)	US	08/377,317	24 January 1995 (24.01.95)	US	08/505,012	21 July 1995 (21.07.95)	US	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KG, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
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<b>(54) Title:</b> RECOMBINANT HUMAN ALPHA-FETOPROTEIN AND USES THEREOF  <b>(57) Abstract</b>  In general, disclosed are methods of inhibiting autoreactive immune cell proliferation in a mammal, involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof; methods of inhibiting a neoplasm in a mammal, involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an anti-neoplasm fragment or analog thereof; and methods of cell culture, involving the use of a media containing recombinant human alpha-fetoprotein or a fragment or analog thereof.																	

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RECOMBINANT HUMAN ALPHA-FETOPROTEIN AND USES THEREOFBackground of the Invention

This invention relates to the expression and  
5 purification of cloned human alpha-fetoprotein; methods  
for treating autoimmune diseases; cancer therapeutics and  
diagnostic methods; and cell growth and cell culture.

Alpha-fetoprotein (AFP) is a serum protein  
normally found at significant levels only in fetal blood.  
10 In adult blood increased alpha-fetoprotein levels are  
associated with liver regeneration and certain  
carcinomas.

Summary of the Invention

In general, the invention features substantially  
15 pure biologically-active recombinant human alpha-  
fetoprotein including a sequence that is substantially  
identical to either amino acids 1 to 389 of Fig. 1 (SEQ  
ID NO: 9) or a fragment thereof; amino acids 198 to 590  
of Fig. 1 (SEQ ID NO: 10) or a fragment thereof; amino  
20 acids 198 to 389 of Fig. 1 (SEQ ID NO: 7) or a fragment  
thereof; amino acids 390 to 590 of Fig. 1 (SEQ ID NO: 8)  
or a fragment thereof; and amino acids 266 to 590 of Fig.  
1 (SEQ ID NO: 11) or a fragment thereof.

In another related aspect, the invention features  
25 a method for using an insect cell for producing  
biologically active recombinant human alpha-fetoprotein  
or a fragment or analog thereof involving

a) providing a transformed insect cell (e.g.,  
Spodoptera frugiperda) including a recombinant DNA  
30 molecule encoding the human alpha-fetoprotein or fragment  
or analog thereof operably linked to an expression  
control element which directs the expression of the human  
alpha-fetoprotein or fragment or analog thereof;

b) culturing the transformed cell; and

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c) recovering the biologically active human alpha-fetoprotein or fragment or analog thereof.

In related aspects, the invention also features substantially pure human alpha-fetoprotein or a fragment  
5 or analog thereof produced using any of the methods described herein, and therapeutic compositions including substantially pure human alpha-fetoprotein (or a fragment or analog thereof) produced using any of the expression systems described herein.

10 In another aspect, the invention features a method of inhibiting autoreactive immune cell proliferation in a mammal (e.g., a human patient), involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an immune cell  
15 anti-proliferative fragment or analog thereof. Such a method is based on my discovery that unglycosylated recombinant human alpha-fetoprotein which is made in a prokaryote (e.g., E. coli) is useful for inhibiting autoreactive immune cells derived from a mammal.

20 Preferably, such immune cells include T cells or B cells; and the recombinant human alpha-fetoprotein (or an immune cell anti-proliferative fragment or analog thereof) used in such methods is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

25 In another aspect, the invention features a method of treating an autoimmune disease in a mammal (e.g., a human patient), involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an immune cell anti-proliferative  
30 fragment or analog thereof. Such an autoimmune disease is multiple sclerosis; is rheumatoid arthritis; is myasthenia gravis; is insulin-dependent diabetes mellitus; or is systemic lupus erythematosus. In other preferred embodiments the autoimmune disease is acquired  
35 immune deficiency syndrome or involves a rejection of a



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transplanted organ, tissue, or cell. Preferably, the recombinant human alpha-fetoprotein used in such methods is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated. In other preferred embodiments, such

5 methods further involve administering to the mammal an immunosuppressive agent in an effective dose which is lower than the standard dose when the immunosuppressive agent is used by itself. Preferably, such an immunosuppressive agent is cyclosporine; is a steroid; is

10 azathioprine; is FK-506; or is 15-deoxyspergualin. In yet another preferred embodiment, such a method involves administering to the mammal a tolerizing agent. Preferably, the recombinant human alpha-fetoprotein used in such methods is produced in a prokaryotic cell (e.g.,

15 E. coli) and is unglycosylated.

According to the invention, administration of recombinant human alpha-fetoprotein ("rHuAFP") (or a fragment or analog thereof) can be an effective means of preventing or treating or ameliorating autoimmune

20 diseases in a mammal. To illustrate this, I have shown that recombinant HuAFP produced in a prokaryotic expression system is effective in suppressing T cell proliferation in response to self antigens, despite the fact that such rHuAFP is not modified in the same fashion

25 as naturally occurring HuAFP. The use of natural HuAFP has heretofore been limited by its unavailability, natural HuAFP is obtained by laborious purification from limited supplies of umbilical cords and umbilical cord serum. Because biologically active rHuAFP can now be

30 prepared in large quantities using the techniques of recombinant DNA, the use of rHuAFP for treating autoimmune diseases is now possible. The use of rHuAFP is especially advantageous since there are no known adverse side effects related to human alpha-fetoprotein

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and it is believed that relatively high doses can be safely administered.

In still other aspects, the invention features compositions and methods for the protection, treatment, and diagnosis of neoplasia, in particular, cancer. This aspect of the invention is based on my discovery that unglycosylated recombinant human alpha-fetoprotein made in a prokaryote (e.g., E. coli) is useful for treating and diagnosing mammals with neoplasms, especially malignant tumors, such as breast or prostate carcinomas, and other carcinomas caused by a proliferation of malignant cells which express receptors which are recognized by recombinant human alpha-fetoprotein.

In one aspect, the invention features a method of inhibiting a neoplasm in a mammal (e.g., a human patient), involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an anti-neoplasm fragment or analog thereof. Preferably, the neoplasm is a malignant tumor (e.g., a breast tumor or a prostate tumor); and the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated. In preferred embodiments, the cells of the neoplasm express a receptor which is recognized by the recombinant human alpha-fetoprotein. Such a neoplasm is generally a carcinoma such as an adenocarcinoma or a sarcoma. In preferred embodiments, the neoplasm proliferates in response to a hormone, e.g, estrogen or androgen. Preferably, administration of recombinant human alpha-fetoprotein inhibits the proliferation of cells of the neoplasm or, alternatively, kills cells of the neoplasm in the mammal. The method further includes administering to the mammal a chemotherapeutic agent.

In another aspect, the invention features a method of protecting a mammal from developing a neoplasm,

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involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is  
5 unglycosylated.

In another aspect, the invention features a hybrid cytotoxin including a recombinant human alpha-fetoprotein (or a fragment or analog thereof) linked to a cytotoxic agent. Examples of such cytotoxic agents include,  
10 without limitation, diphtheria toxin, Pseudomonas exotoxin A; ricin and other plant toxins such as abrin, modeccin, volkensin, viscumin; cholera toxin (produced by Vibrio cholerae bacteria); the so-called "Shiga-like" toxins (produced by E. coli and other enteric bacteria);  
15 Salmonella heat-labile enterotoxin; and E. coli heat-labile enterotoxin. In other preferred embodiments, the cytotoxic agent is non-proteinaceous. Examples of such non-proteinaceous cytotoxic agents include, without  
20 limitation, anti-cancer agents such as doxorubicin, as well as  $\alpha$ -emitting radionuclides such as astatine and  $\beta$ -emitting nuclides such as yttrium. Preferably, the cytotoxic agent of the hybrid cytotoxin is linked by a peptide bond to the recombinant human alpha-fetoprotein, and the hybrid toxin is produced by expression of a  
25 genetically engineered hybrid DNA molecule. In other preferred embodiments, the cytotoxic agent of the hybrid cytotoxin is a protein; such a cytotoxic agent is chemically conjugated to the recombinant human alpha-fetoprotein.

30 In other aspects, the invention features a detectably-labelled recombinant human alpha-fetoprotein or a detectably-labelled fragment or analog thereof capable of binding to a human neoplastic cell. Preferably such a molecule is labelled with a  
35 radionuclide, e.g., technetium-99m, iodine-125, iodine-

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131, or indium. Other detectable labels include, without limitation, enzymes, fluorophores, or other moieties or compounds which emit a detectable signal (e.g., radioactivity, fluorescence, color) or emit a detectable  
5 signal after exposure of the label to its substrate or, alternatively, the detectable signal can be an epitope recognized by an antibody (e.g., an epitope of alpha-fetoprotein or an epitope which is specifically engineered into the recombinant alpha-fetoprotein such as  
10 the HA or myc epitopes). Preferably, the molecule targets a malignant tumor (e.g. a breast tumor, a prostate tumor, or a carcinoma) which expresses a receptor which is recognized by the recombinant human alpha-fetoprotein (or fragment or analog thereof).  
15 Typically, such recombinant alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

Detectably-labelled recombinant human alpha-fetoprotein (or is a fragment or analog thereof) is  
20 useful for methods of imaging a neoplastic cell-containing region in a human patient in vivo. In general, the method involves: (a) providing a detectably-labelled molecule of recombinant human alpha-fetoprotein (or a fragment or analog thereof); (b) administering the  
25 molecule to the patient; (c) allowing the labelled molecule to bind and allowing unbound molecule to be cleared from the region; and (d) obtaining an image of the neoplastic cell-containing region. Preferably, the region is the breast or is the prostate. In other  
30 preferred embodiments, the region, without limitation, is liver tissue, is lung tissue, is spleen tissue, is pancreatic tissue, is brain tissue, is lymph tissue, or is bone marrow. Preferably, the image is obtained using dynamic gamma scintigraphy.

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Detectably-labelled recombinant human alpha-fetoprotein (or a fragment or analog thereof) can also be used in a method for diagnosing a neoplasm in a mammal (e.g., a human patient). Such a method includes: (a) 5 contacting the biological sample with the detectably-labelled molecule of recombinant human alpha-fetoprotein; and (b) detecting the label bound to the sample, where the detection of label above background levels is indicative that the patient has a neoplasm. Preferably, 10 the method involves a biological sample including cells fixed and sectioned prior to the contacting step, and the label bound to the sample is bound to areas corresponding to the cell membrane of the cells. In preferred embodiments, the biological sample is from the breast or 15 prostate of a human patient.

Detectably-labelled recombinant human alpha-fetoprotein (or fragment or analog thereof) can also be used in a method for detecting a neoplasm a mammal in vivo. Such a method includes: (a) administering a 20 diagnostically effective amount of the detectably-labelled molecule of recombinant human alpha-fetoprotein; and (b) detecting the presence of the detectable label bound to a tissue of the mammal, where an amount of label above background levels is indicative of the presence of 25 the neoplasm in the mammal.

In preferred embodiments, the method involves a human patient suspected of having a breast cancer, and the tissue is breast tissue. In other preferred embodiments, the method involves a human patient suspected of having a 30 prostate cancer, and the tissue is prostate tissue. Preferably, the detectably labelled recombinant human alpha-fetoprotein is linked to a radionuclide (e.g., technetium-90) and the detection step is accomplished by radioimaging (e.g., dynamic gamma scintigraphy).

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In another aspect, the invention features kits for detecting a neoplasm or any cell expressing a receptor which is recognized by recombinant human alpha-fetoprotein (or a fragment or analog thereof) in vivo, in situ or in vitro. In general, the kits include a recombinant human alpha-fetoprotein which is recognized by a neoplasm, and which may be detectably labeled. If the recombinant human alpha-fetoprotein is unlabelled, a second reagent containing a detectable label (e.g. a radionuclide such as technetium-90, iodine-125, iodine-131, or indium) is preferably included. Where the detectable label is an enzyme, the kit further includes a substrate reagent for the enzyme. The kit may also include a reagent for linking the detectable label to the recombinant alpha-fetoprotein. In another embodiment, the kit for detecting a neoplasm or any unwanted cell expressing a receptor which is recognized by recombinant human alpha-fetoprotein (or a fragment or analog thereof) includes a reagent containing an antibody which specifically binds the recombinant human alpha-fetoprotein and a reagent including a detectably labeled recombinant human alpha-fetoprotein that is specifically bound by the anti-alpha-fetoprotein antibody. Preferably, the recombinant human alpha-fetoprotein of the kit is produced in a prokaryotic cell (E. coli) and is unglycosylated.

The use of recombinant human alpha-fetoprotein for the treatment and diagnosis of cancer offers a number of advantages. For example, rHuAFP can be administered directly to a tumor site. Recombinant HuAFP can also be chemically defined and synthesized, and prepared in large quantities using the techniques of recombinant DNA, e.g., those which are described herein. Moreover, unlike conventional cancer chemotherapies and radiotherapies, recombinant human alpha-fetoprotein causes minimal side

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effects such as nausea, vomiting, and neurotoxicity. Accordingly, relatively high doses of rHuAFP can be safely administered.

The diagnostic methods of the invention are  
5 advantageous since they allow for rapid and convenient diagnosis of a neoplasm. For example, the use of rHuAFP as a diagnostic agent (e.g., by radioimaging using scintigraphy) is especially advantageous for real time  
10 imaging of cancer for pre-surgical or intraoperative localization and for staging of a cancer, e.g., breast cancer, as well as during post-surgical examinations. The use of such diagnostic procedures permits non-invasive determination of the presence, location, or absence of a neoplasm which is advantageous for  
15 monitoring the condition of a patient.

In still other aspects, the invention features a cell culture medium including recombinant human alpha-fetoprotein or a cell-stimulating fragment or analog thereof. This aspect of the invention is based on my  
20 discovery that unglycosylated recombinant human alpha-fetoprotein made in a prokaryote (e.g., E. coli) is a cell proliferative agent, e.g., promotes the growth of bone marrow in vitro. Preferably, such recombinant human alpha-fetoprotein is produced in a prokaryotic cell (E.  
25 coli) and is unglycosylated.

Accordingly, this aspect of invention features a method of cell culture, the method including (a) providing a cell culture medium including recombinant human alpha-fetoprotein; (b) providing a cell; (c) and  
30 growing the cell in the medium, where the cell proliferates, and is maintained. Preferably, the cell is a mammalian cell. Examples of such mammalian cells include bone marrow cells (e.g., T cells, natural killer cell, lymphocyte), hybridomas, or a genetically-  
35 engineered cell line. Examples of other cells include

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hematopoietic cells such as stem cells, blast cells, progenitor cells (e.g., erythroid progenitor cells such as burst-forming units and colony-forming units), myeloblasts, macrophages, monocytes, macrophages, lymphocytes, T-lymphocytes, B-lymphocytes, eosinophils, basophils, tissue mast cells, megakaryocytes (see e.g., *Best and Taylor's Physiological Basis of Medical Practice*, John B. West, ed., Williams & Wilkins, Baltimore). In other preferred embodiments the method involves ex vivo cell culture.

In another aspect, the invention features a method for inhibiting myelotoxicity in a mammal (e.g., a human patient) involving administering to the mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or a myelotoxic-inhibiting analog or fragment thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (E. coli) and is unglycosylated.

In another aspect, the invention features a method of inhibiting suppression of bone marrow cell proliferation in a mammal, the method involving administering to the mammal an effective amount of recombinant alpha-fetoprotein or an anti-suppressive fragment or analog thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

In another aspect, the invention features a method of promoting bone marrow cell proliferation in a mammal, involving administering to the mammal an effective amount of recombinant human alpha-fetoprotein or a cell-stimulating fragment or analog thereof. Preferably, the recombinant human alpha-fetoprotein is produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

In another aspect, the invention features a method of preventing bone marrow cell transplantation rejection



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in a mammal, involving administering to the mammal an effective amount of recombinant human alpha-fetoprotein or an anti-rejection fragment or analog thereof.

Preferably, the recombinant human alpha-fetoprotein is  
5 produced in a prokaryotic cell (e.g., E. coli) and is unglycosylated.

According to the methods of the invention, administration of rHuAFP (or a fragment or analog thereof) can be an effective means for promoting and boosting cell growth in vitro, ex  
10 vivo, or in vivo. Additionally, administration of the compounds of the invention can also be an effective means of preventing or treating or ameliorating myelotoxemia in a mammal.

The use of rHuAFP (or a fragment or analog  
15 thereof) as a principal component of tissue culture media is advantageous since there is little potential for contamination with pathogens.

By "human alpha-fetoprotein" is meant a polypeptide having substantially the same amino acid  
20 sequence as the protein encoded by the human alpha-fetoprotein gene as described by Morinaga et al., Proc. Natl. Acad. Sci., USA 80: 4604 (1983). The method of producing recombinant human alpha-fetoprotein in a prokaryotic cell is described in U.S. Pat. No. 5,384,250,  
25 and according to the methods described herein.

By "expression control element" is meant a nucleotide sequence which includes recognition sequences for factors that control expression of a protein coding sequence to which it is operably linked. Accordingly, an  
30 expression control element generally includes sequences for controlling both transcription and translation, for example, promoters, ribosome binding sites, repressor binding sites, and activator binding sites.

By "substantially the same amino acid sequence" is  
35 meant a polypeptide that exhibits at least 80% homology

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with naturally occurring amino acid sequence of human alpha-fetoprotein, typically at least about 85% homology with the natural human alpha-fetoprotein sequence, more typically at least about 90% homology, usually at least 5 about 95% homology, and more usually at least about 97% homology with the natural human alpha-fetoprotein sequence. The length of comparison sequences will generally be at least 16 amino acids, usually at least 20 amino acids, more usually at least 25 amino acids, 10 typically at least 30 amino acids, and preferably more than 35 amino acids.

Homology, for polypeptides, is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, 15 University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Protein analysis software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative 20 substitutions typically include substitutions within the following groups: glycine alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

25 As used herein, the term "substantially pure" describes a protein or polypeptide which has been separated from components which naturally accompany it. Typically, a protein of interest is substantially pure when at least 60% to 75% of the total protein in a sample 30 is the protein of interest. Minor variants or chemical modifications typically share the same polypeptide sequence. A substantially pure protein will typically comprise over about 85 to 90% of the protein in sample, more usually will comprise at least about 95%, and 35 preferably will be over about 99% pure. Normally, purity

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is measured on a chromatography column, polyacrylamide gel, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from the native contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Thus the term can be used to describe polypeptides and nucleic acids derived from eukaryotic organisms which have been synthesized in E. coli and other prokaryotes.

The present invention provides for substantially pure human alpha-fetoprotein. Various methods for the isolation of human AFP from biological material may be devised, based in part upon the structural and functional properties of human alpha-fetoprotein. Alternatively, anti-AFP antibodies may be immobilized on a solid substrate to generate a highly specific affinity column for purification of human AFP.

Besides substantially full-length polypeptides, the present invention provides for biologically active recombinant fragments or analogs of human alpha-fetoprotein. For example, fragments active in ligand binding or immunosuppression.

The natural or synthetic DNA fragments coding for human alpha-fetoprotein or a desired fragment thereof will be incorporated into DNA constructs capable of introduction into and expression in cell culture. DNA constructs prepared for introduction into such hosts will typically include an origin of replication which can be utilized by the host cell, a DNA fragment encoding the desired portion of human alpha-fetoprotein, transcription and translational initiation regulatory sequences operably linked to the alpha-fetoprotein encoding

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segment, and transcriptional and translational termination regulatory sequences operably linked to the alpha-fetoprotein encoding segment. The transcriptional regulatory sequences will typically include a

5 heterologous promoter which is recognized by the host. The selection of an appropriate promoter will depend upon the host, but promoters such as the trp, tac and phage promoters, tRNA promoters and glycolytic enzyme promoters may be used under appropriate circumstances (Sambrook et

10 al. eds., *Molecular Cloning: Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY 1989). In some instances it may be desirable to include appropriately positioned recognition sequences for factors capable of regulating transcription in the host

15 cell (e.g., the lac repressor of E. coli). Commercially available expression vectors, which include the replication system and transcriptional and translational regulatory sequences together with convenient sites for the insertion of a DNA fragment encoding the gene to be

20 expressed may be used.

The various promoters, transcriptional, and translational described above are generally referred to as an "expression control element."

It is also possible to integrate a DNA fragment

25 encoding all or part of human AFP into the host cell's chromosome.

The vectors containing the DNA segments of interest can be transferred into the host cell by well-known methods, which vary depending on the type of

30 cellular host (Sambrook et al., supra). The term "transformed cell" is meant to also include the progeny of a transformed cell.

Prokaryotic hosts useful for high level expression of recombinant proteins include: various strains of E.

35 coli, Bacillus subtilis, and Pseudomonas.

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The method of the invention provides a means by which to generate large quantities of human alpha-fetoprotein having biological activity. AFP produced according to the method of the invention has biological activity despite the fact that it is not modified in the same fashion as naturally occurring human AFP.

By "immune cell anti-proliferative" is meant capable of inhibiting the growth of an undesirable immune cell (e.g., an autoreactive T cell as measured using the assays described herein).

By "neoplasm" is meant any unwanted growth of cells serving no physiological function. In general, a cell of a neoplasm has been released from its normal cell division control, i.e., a cell whose growth is not regulated by the ordinary biochemical and physical influences in the cellular environment. In most cases, a neoplastic cell proliferates to form a clone of cells which are either benign or malignant. Examples of neoplasms include, without limitation, transformed and immortalized cells, tumors, and carcinomas such as breast cell carcinomas and prostate carcinomas.

By "therapeutically effective amount" is meant a dose of unglycosylated recombinant human alpha-fetoprotein or an anti-neoplasm fragment or analog thereof capable of inhibiting the proliferation of a neoplasm or is capable of inhibiting autoreactive immune cell proliferation or stimulating the proliferation of a cell (e.g., a bone marrow cell).

By "diagnostically effective amount" is meant a dose of detectably-labelled recombinant human alpha-fetoprotein or a detectably-labelled fragment or analog thereof that can be detected within a targeted region in a mammal (e.g., a human patient).

By "cell-stimulating" is meant increasing cell proliferation, increasing cell division, promoting cell

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differentiation and/or development, or promoting cell longevity.

By "myelotoxic-inhibiting" is meant inhibiting myeloablation.

- 5 Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Detailed Description

The drawings will first be described.

#### 10 Drawings

Fig. 1 is the nucleotide sequence (SEQ ID NO: 4) and deduced amino acid sequence (SEQ ID NO: 5) of the cDNA encoding human alpha-fetoprotein.

- Fig. 2 is the 10% SDS-PAGE analysis of rHuAFP  
15 Fragment I (SEQ ID NO: 11) (Lane A, MW marker; Lane B, natural human alpha-fetoprotein (AFP); Lane C, unpurified rHuAFP; Lane D, rHuAFP Fragment I, and Lane E, rHuAFP (amino acids 1- 590 of Fig. 1, SEQ ID NO: 5).

- Fig. 3 is a bar graph showing the inhibition of  
20 human AMLR by E. coli-derived rHuAFP and domain fragments.

- Fig. 4 is a series of graphs (Figs. 4A-4D) showing the purity and biochemical characteristics of baculovirus- and E. coli-derived rHuAFP using  
25 polyacrylamide gel electrophoresis and column chromatography. Fig. 4A is a 10% non-denaturing alkaline polyacrylamide gel showing the purity of rHuAFP. Mouse amniotic fluid proteins (transferrin, AFP and albumin) are shown in lane 1, natural HuAFP (lane 2), baculovirus-  
30 derived rHuAFP (lane 3), and E. coli-derived rHuAFP (lane 4). Fig. 4B is a 10% sodium dodecyl sulfate-polyacrylamide gel showing the purity of rHuAFP produced using baculovirus and E. coli expression systems. Molecular weight markers are shown in lane 1, natural

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HuAFP, baculovirus- and E. coli-derived rHuAFP are shown in lanes 2, 3, and 4, respectively. Fig. 4C is a series of FPLC chromatograms of natural HuAFP, baculovirus-derived and E. coli-derived rHuAFP eluted on a MonoQ anion exchange column. The superimposed chromatograms identify natural HuAFP (Chromatogram 1), baculovirus- and E. coli-derived rHuAFP (Chromatograms 2 and 3, respectively). Fig. 4D is a series of HPLC chromatograms obtained by passing 50  $\mu$ g of natural and rHuAFP by passing through a reverse phase Delta Pak C18 column (Waters) and eluting with a gradient of 0-100% acetonitrile in 0.1% TFA. The superimposed chromatograms identify natural HuAFP (Chromatogram 1) and baculovirus- and E. coli-derived rHuAFP (Chromatograms 2 and 3, respectively).

Fig. 5 is a bar graph showing that monoclonal anti-natural HuAFP antibodies block immunosuppression of the AMLR by rHuAFP produced using baculovirus and E. coli expression systems. Immunosuppression by rHuAFP produced using baculovirus and E. coli expression systems was significant ( $p < 0.002$ ) and blocking of rHuAFP-mediated immunosuppression by the AMLR by monoclonal anti-natural HuAFP ( $\alpha$  AFP) antibodies was also significant ( $p < 0.03$ ). AMLR cultures were set up with  $2 \times 10^5$  responding T cells with  $2.5 \times 10^5$  irradiated autologous non-T cells in the presence or absence of protein, harvested at 144 hours, and autoproliiferation was measured by the amount of  $^3\text{H}$ -thymidine incorporated by autoreactive T cells. Blocking of the autoproliiferative effects of rHuAFP was carried out by adding murine anti-human AFP monoclonal antibodies at a dilution of 1/8 (125  $\mu$ g/ml) to AMLR cultures suppressed by 100  $\mu$ g/ml baculovirus derived (diagonal bars) and by 100  $\mu$ g/ml of E. coli-derived (open bars) rHuAFP. Control cultures consisted of the AMLR in the

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presence of 1/8 dilution of anti-human AFP (à AFP) monoclonal antibodies.

Fig. 6 is a series of bar graphs (Figs. 6A and 6B) showing the effects of rHuAFP-mediated immunosuppression using human AMLR (Fig. 6A) and PBL (Fig. 6B) assays. Fig. 6A shows the results of autologous mixed lymphocyte reaction (AMLR) prepared by co-culturing 250,000 T cells with an equivalent amount of autologous irradiated non-T lymphocytes. Recombinant HuAFP preparations derived from *E. coli* and baculovirus expression systems and albumin were added at a concentration of 100 µg/ml at the initiation of culture. Proliferative responses were measured at 144 hours by <sup>3</sup>H-thymidine incorporation. Fig. 6B shows the results of PBLs (2 x 10<sup>5</sup>) stimulated with 1 µg/ml ConA which were cultured in RPMI medium supplemented with only 2 mg/ml albumin for 48 hrs. Albumin and rHuAFP derived from *E. coli* and baculovirus were added to the initiation of the cultures at a concentration of 100 µg/ml. Proliferative responses were measured as the amount of <sup>3</sup>H-thymidine incorporated during DNA synthesis. The SEM were determined to represent less than 5% of the value of the mean.

Fig. 7 is a plasmid map of pVT-PlacZ.

Fig. 8 is a series of graphs showing the inhibitory effects of the rHuAFP on kinetics of T cell activation (Fig. 8A) and the dose-response relationship of rHuAFP on autoproliiferating T cells (Fig. 8B). Fig. 8A is a graph showing proliferative responses over a 4 day time course of cells cultured in the absence (▽) and in the presence of 100 µg/ml (▼) rHuAFP. (●) denotes the background proliferation of the responder cell population cultured separately. Recombinant HuAFP-mediated suppression on the AMLR over the time course was significant (p<0.01). Fig. 8B is a graph showing the inhibition of autoproliiferating T cells at 144 hours with



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amounts of rHuAFP ranging from 6-100  $\mu\text{g/ml}$  ( $\nabla$ ). ( $\nabla$ ) denotes the control response of the reaction in the absence of protein. Inhibition of autoreactive T cells by rHuAFP in the range of 12.5-100  $\mu\text{g/ml}$  is significant ( $p < 0.005$ ).

Fig. 9 is a bar graph showing the effect of rHuAFP on estrogen-stimulated post-confluent growth of MCF-7 breast cancer cells.

Fig. 10 is a bar graph showing murine bone marrow proliferation in serum-free RPMI medium in the presence or absence of both 400  $\mu\text{g/ml}$  rHuAFP and 5  $\mu\text{g/ml}$  transferrin.

Expression of Recombinant Human Alpha-Fetoprotein  
Construction of a cDNA Library

A cDNA library was constructed with size-fractionated cDNA (0.5-3 kb) prepared from poly(A)<sup>+</sup> RNA isolated from liver cells (~3 grams wet weight) of a 4.5 months old human abortus. (Alternatively, a fetal cDNA library may be obtained from Clontech Laboratories, Inc., Palo Alto, CA.) Total RNA was prepared by the guanidium thiocyanate method (Chirgwin et al., Biochemistry 18:5294, 1979), and mRNA was selected by oligo(dT)-cellulose chromatography (Collaborative Research, Bedford, MA) (Current Protocols in Molecular Biology, Ausubel et al., eds., Wiley Interscience, New York, 1989). cDNA was synthesized using the Librarian II cDNA synthesis kit (Invitrogen, San Diego, CA) and fractionated on a 1% agarose gel. Fragments of 0.5 to 3 kb were extracted and ligated to vector pTZ18-RB (Invitrogen), and used to transform competent E. coli DH1 $\alpha$ F' (Invitrogen). Colony lifts were performed with Colony/Plaque Screen filters (DuPont, Wilmington, DE), and the transferred bacterial colonies were lysed and denatured by incubation in a solution of 0.5M NaOH, 1.5M NaCl for 10 min. The filters were washed for 5 min in

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1.5M NaCl, 0.50M Tris-HCl (pH 7.6), and air dried. Filters were then washed 5 times in chloroform, soaked in 0.3M NaCl to remove cellular debris, and then air dried. The DNA was fixed to the nitrocellulose by baking at 80°  
5 under vacuum for 2 hrs. The baked filters were prehybridized for 3 hr at 37°C in 6X SSC (1X SSC = 150mM NaCl, 15mM sodium citrate [pH 7.0]), 1X Denhardt's solution (0.2 g/l polyvinylpyrrolidone, 0.2 g/l BSA, 0.2 g/l Ficoll 400), 0.05% sodium pyrophosphate, 0.5 % SDS,  
10 and 100 µg/ml *E. coli* DNA. Hybridization was performed for 18-24 hr at 37°C in the same solution without SDS, containing 1-2 x 10<sup>6</sup> cpm/ml of two oligonucleotides <sup>32</sup>P-labelled by 5'-end phosphorylation (*Current Protocols in Molecular Biology, supra*). The sequence of the  
15 oligonucleotides used for probing the library: 5'-TGCTGTCAGGATGGGGAAAAA-3' (SEQ ID NO: 1) and 5'-CATGAAATGACTCCAGTA-3' (SEQ ID NO: 2), correspond to positions 772 to 792 and positions 1405 to 1422 of the human AFP coding sequence respectively. Filters were  
20 washed twice for 30 min at 37°C with 6X SSC, 0.05% sodium pyrophosphate and once for 30 min at 48°C in the same solution. Dried filters were exposed to Kodak XAR films in the presence of Du Pont Cronex Lightning Plus intensifier screens for 24-48 hr to identify positive  
25 clones. Positive clones were isolated, amplified, and subjected to Southern blot analysis (*Current Protocols in Molecular Biology, supra*). Briefly, purified DNA was hydrolyzed with the appropriate restriction enzymes, and the resulting fragments were resolved on a 1% agarose  
30 gel. The DNA was then transferred to a nitrocellulose membrane. Hybridization conditions were as described above except that a third <sup>32</sup>P-labelled oligonucleotide (5'-CATAGAAATGAATATGGA-3' (SEQ ID NO: 3), representing positions 7 to 24 of the human AFP coding region) was  
35 used in addition to the other two probes described above.

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Five positive clones were identified among the 3,000 colonies screened. One clone, pLHuAFP, was used in the construction described below.

Construction of Full Length Human AFP cDNA

5 A construct containing a translation initiation codon followed by the human AFP coding sequence and a translation termination codon was created using the following five DNA fragments.

Fragment 1: Two unphosphorylated oligonucleotides  
10 were annealed to form a double-stranded DNA molecule consisting of a 5'-end cohesive EcoRI recognition site, followed by an ATG initiation codon and the first 60 bp of the human AFP cDNA up to and including the PstI restriction site located at position 60 in the coding  
15 sequence (In this scheme, nucleotide 1 is the first nucleotide of the first codon (Thr) in the mature protein and corresponds to nucleotide 102 of Morinaga et al., supra). This fragment was ligated to pUC119 (pUC19 with the intergenic region of M13 from HgiA I at 5465 to AhaII  
20 at 5941 inserted at the Nde I site of pUC19) linearized with Eco RI and Pst I. The resulting DNA was amplified in E. coli NM522 (Pharmacia, Piscataway, NJ) The EcoRI-PstI insert was recovered by enzymatic digestion of the recombinant plasmid followed by electrophoretic  
25 separation on a 5% polyacrylamide gel and isolation from the gel.

Fragment 2: A 97 bp human AFP cDNA fragment (positions 57 to 153) was obtained by digesting pLHuAFP with PstI and NsiI and gel purifying as described above.  
30 This clone contains the entire coding region of human AFP as well as 5' and 3' untranslated sequences.

Fragment 3: A 224 bp human AFP cDNA fragment (positions 150 to 373) was obtained by digesting pLHuAFP with NsiI and AlwNI and purifying as described above.

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Fragment 4: A 1322 bp human AFP cDNA fragment (positions 371 to 1692) was obtained by digesting pLHuAFP with AlwNI and StyI and purifying as described above.

Fragment 5: Two unphosphorylated oligonucleotides  
5 were annealed to form a 86 bp double-stranded DNA  
contains the human AFP sequence from position 1693 in the  
StyI site to the TAA termination codon that ends the AFP  
coding region at position 1773, followed by a cohesive  
BamHI site. This synthetic DNA was used without any  
10 further manipulations.

pBlueScript (StrataGene, La Jolla, CA) was  
completely hydrolyzed with EcoRI and BamHI, and added to  
ligation mixture containing the five purified fragments  
described above. A control ligation contained only the  
15 linearized pBluescript. Portions of these two ligation  
mixtures were used to transform competent E. coli DH5 $\alpha$   
(GIBCO/BRL, Grand Island, NY). Recombinant plasmids were  
isolated from several transformants and screened by  
extensive restriction enzyme analysis and DNA sequencing.  
20 One recombinant plasmid was selected and termed pHuAFP.  
It was used for subsequent insertion of the human AFP  
gene into several expression vectors. pHuAFP includes a  
unique EcoRI-BamHI fragment that contains the complete  
coding sequence for human AFP in addition to an ATG start  
25 codon at the 5'-end and a TAA stop codon at the 3'-end.

#### AFP Expression Vectors

Successful high-level synthesis of human AFP in  
E. coli was achieved in three different expression  
systems. The TRP system gave direct expression. The RX1  
30 system yielded a fusion protein containing 20 amino acids  
encoded by trpE and vector sequences. The MAL system  
expressed AFP fused to the malE gene product, a 42 kd  
maltose-binding protein.

TRP Expression System: The 1186 bp EcoRI-BamHI  
35 AFP encoding fragment of pHuAFP was cloned into the

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expression vector pTrp4 (Olsen et al., J. Biotechnol. 9:179, 1989) downstream of the trp promoter and a modified ribosome-binding site.

Briefly, pHuAFP was digested with EcoRI and BamHI, 5 and the ends were filled using Klenow polymerase. The 1186 bp AFP fragment was then gel purified. pTrp4 was ClaI digested, the ends were filled using Klenow polymerase, and the linearized vector was gel purified. The 1186 bp AFP fragment and pTrp4 backbone were ligated 10 and used to transform competent E. coli of the following strains: DH5 $\alpha$ , BL21 (F.W. Studier, Brookhaven National Laboratory, Upton, NY), SG927 (American Type Culture Collection, Rockville, MD: Acc. No. 39627), SG928 (ATCC Acc. No. 39628), and SG935 (ATCC Acc. No. 39623).

15 RX1 Expression System: Human AFP cDNA was cloned into the expression vector pRX1 (Rimm et al., Gene 75:323, 1989) adjacent to the trp promoter and in the translation frame of TrpE. The human AFP cDNA was excised from pHuAFP by digestion with EcoRI and BamHI and 20 cloned into suitably treated pRX1 (BioRad Laboratories, Hercules, CA). The E. coli strains described above and CAG456 (D.W. Cleveland, Johns Hopkins University, Baltimore, MD) were then transformed with the final plasmid construction identified as pRX1/HuAFP.

25 MAL Expression System: AFP cDNA was inserted into the expression vector pMAL (New England Biolabs, Inc., Beverly, MA) under control of the tac promoter and in the translation frame of MalE. Briefly, pHuAFP was hydrolysed with BamHI and the ends made blunt using 30 Klenow polymerase. The human AFP cDNA was released from the rest of the plasmid DNA by EcoRI digestion and then gel purified. The purified fragment was ligated to appropriately digested pMAL-C. A correctly oriented recombinant plasmid, designated pMAL/HuAFP, was used to

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transform E. coli DH5 $\alpha$ , TBI (New England Biolabs) and SG935.

The AFP coding region used in the construction of the three expression vectors was sequenced and found to  
5 encode full length AFP.

Expression of AFP in E. coli

Bacterial cultures were incubated at 30°C or 37°C with aeration. Overnight cultures of E. coli were grown in LB medium supplemented with the appropriate  
10 antibiotics as required (Tetracycline-HCl was at 50  $\mu$ g/ml, and ampicillin-Na was at 100  $\mu$ g/ml).

TRP and RX1 Expression Systems: The trp promoter was induced under tryptophan starvation conditions. Induction was performed in M9CA medium prepared as  
15 follows: 1 g Casamino acids (Difco Laboratories, Detroit, MI), 6 g Na<sub>2</sub>HPO<sub>4</sub>, 3g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g NaCl, 1 g NH<sub>4</sub>Cl is added to one liter milli-Q water (Millipore Corp., Bedford, MA), the pH adjusted to 7.4 and the solution autoclaved. The cooled medium is made 2mM MgSO<sub>4</sub>, 0.1mM  
20 CaCl<sub>2</sub>, and 0.2% glucose. After a 100-fold dilution of an overnight culture in M9CA supplemented with antibiotics, the cells were grown at 30°C to A<sub>550</sub> of 0.4, harvested by centrifugation, and stored as pellets at -20°C.

MAL Expression System: The tac promoter was  
25 induced with the gratuitous inducer IPTG. Overnight cultures were diluted 100-fold in LB medium supplemented with antibiotics, and the cells grown at 37°C to A<sub>550</sub> of 0.4. IPTG was then added to a final concentration of 0.3mM, and the bacteria incubated an additional 2 hr.  
30 The cells were then harvested by centrifugation and stored as pellets at -20°C.

Detection of AFP Expressed in E. Coli

Analytical studies were performed to determine the expression and behavior of recombinant AFP. Cell pellets  
35 were either suspended in SDS-lysis solution (0.16M Tris-

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HCl [pH 6.8], 4% w/v SDS, 0.2M DTT, 20% glycerol, 0.02% bromophenol blue), boiled for 5 min, and used for analysis by SDS-PAGE or suspended in a lysis buffer consisting of 10 mM  $\text{Na}_2\text{HPO}_4$ , 30mM NaCl, 0.25% Tween 20, 5 10mM EDTA, 10mM EGTA and incubated with 1 mg/ml lysozyme at 4°C for 30 min prior to sonication in pulse mode for 3 x 1 min at 50% power (Sonics and Materials, Danbury, CT: model VC300 sonifier). The lysate was centrifuged at 10,000g for 20 min, and the supernatant containing 10 soluble protein was decanted in a separate test tube and frozen at -20°C until used. The pellet containing insoluble protein was resuspended in SDS-lysis buffer, boiled for 5 min and kept at -20°C until used. Total protein released in SDS-lysis buffer, as well as soluble 15 and pellet fractions were analyzed by SDS-PAGE and immunological detection following western blot transfer. In these studies Coomassie blue stained gels were routinely scanned with a video densitometer (BioRad, model 620). This allowed a qualitative assessment of the 20 amount of recombinant AFP produced as a percentage of total cellular protein.

#### Purification of AFP Expressed in the TRP System

All procedures were carried out at 4°C, unless otherwise stated. Each frozen cell pellet from a one 25 liter culture was resuspended in 25 ml of lysis buffer A, 50mM Tris-HCl [pH 7.5], 20% sucrose, 100 µg/ml lysozyme, 10 µg/ml PMSF), and incubated for 10 min. EDTA was added to a final concentration of 35mM, and the extract allowed to stand a further 10 min. Following the addition of 25 30 ml of lysis buffer B (50mM Tris-HCl [pH 7.5], 25mM EDTA, 0.2% Triton X-100), the lysate was incubated an additional 30 min. The cell lysate was centrifuged at 12,000g for 20 min, and the precipitate containing the recombinant AFP was washed twice with 50 ml of wash 35 buffer (50mM Tris-HCl [pH 8.0], 10mM EDTA, 0.2% Triton X-

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100), followed each time by centrifugation as above. The precipitate was dissolved in 50 ml of denaturation buffer (0.1M  $K_2HPO_4$  [pH 8.5], 6M guanidine-HCl, 0.1M 2-mercaptoethanol), sonicated, and then mixed on a Nutator 5 (Clay Adams) for 4 hr. The solubilized extract was diluted 50-fold in 50mM Tris-HCl, 100mM NaCl, 1mM EDTA, and the recombinant AFP protein allowed to renature for 24 hr. This 50-fold dilution step is important because prior to dilution AFP appears to be microaggregated. 10 Subsequent to dilution and reconcentration, AFP is not aggregated. The solution was concentrated 100-fold on YM10 membranes using an Amicon filtration unit, and clarified through a Millex 0.22  $\mu$ m membrane filter (Millipore). The recombinant AFP was further purified at 15 room temperature on a Mono Q column (Pharmacia) equilibrated in 20mM Tris-HCl (pH 8.0) with bound proteins eluted using a linear gradient of 0-100% 1M NaCl, 20mM Tris-HCl (pH 8.0). Fractions were analyzed by SDS-PAGE, APAGE, and Western blotting. 20 These general techniques of polypeptide expression and purification can also be used to produce and isolate useful human alpha-fetoprotein fragments or analogs (described below).

#### Polyacrylamide Gel Electrophoresis and Western

#### 25 Immunodetection Procedures

SDS-PAGE in discontinuous buffer system and alkaline-PAGE were performed according to Hames et al. (*Gel Electrophoresis of Proteins: A Practical Approach*, IRL Press, London, 1981) using the mini-Protean 30 electrophoresis apparatus (BioRad). Immunological detection of recombinant human AFP following SDS-PAGE or APAGE was accomplished by soaking the gels in transfer buffer (12.5mM Tris-HCl, 96mM glycine, 20% methanol [pH 8.2]) for 15 min. Individual gels were then layered with 35 an Immobilon PVDF membrane (Millipore) and sandwiched



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between the two electrode grids of the mini-Protean transfer device (BioRad), with the gels adjacent to the cathode. The system was immersed in transfer buffer, and a 150 mA current was applied for 2 hr. Unreacted sites  
5 on the Immobilon PVDF sheets were blocked in 20mM Tris-HCl (pH 7.5), 500mM NaCl, 3% gelatin for 1 hr. Rabbit anti-human AFP antiserum and goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase (BioRad) were used as the primary and the secondary antibodies,  
10 respectively. The alkaline phosphatase activity was detected using 5-brome-4-chloro-3-indolyl phosphate and p-nitroblue tetrazolium (Bio-Rad).

#### Quantitation of AFP Expression

Recombinant human AFP was quantitated using a  
15 human AFP ELISA kit (Abbott Laboratories, Chicago, IL).

AFP yield was estimated by scanning silver stained gels. When SG935 cells are transformed with the AFP encoding plasmid that employs the Trp expression system, AFP represents 2 to 5% of total cellular E. coli protein  
20 (approximately 3-7 mg AFP per liter of culture). As described above, most AFP in the initial extract is insoluble. The above-described resolubilization procedure permits 50-60% recovery of AFP in the form of stable, semi-purified, monomeric AFP (approximate yield  
25 50 mg/20 l of E. coli). This can be further purified to yield 25 mg of pure monomeric AFP.

#### N-Terminal Analysis

Automatic Edman degradations were performed using a Porton protein/peptide gas phase microsequencer with an  
30 integrated customized microbore HPLC to optimize sequence. Protein sequence analysis was aided by the use of selected programs within the PC/Gene software package (Intelligenetics).

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Cloning, Expression, and Purification of HuAFP Using A  
Baculovirus Expression System

Recombinant baculovirus expressing HuAFP (or a fragment or analog thereof) is constructed according to standard methods known in the art (see, e.g., U.S. Pat. No. 4,745,051). This process generally involves two steps. The gene to be expressed, e.g., rHuAFP or a fragment or analog thereof (described *infra*), is first cloned into a plasmid transfer vector downstream from a baculovirus promoter that is flanked by baculovirus DNA derived from a nonessential locus, e.g., the polyhedrin gene. This plasmid is then introduced into insect cells along with circular wild-type genomic DNA for homologous recombination to occur. Resulting recombinant progeny are then screened, e.g., using sequential plaque assays to purify recombinant virus away from the nonrecombinant parental strain. Viral amplification is also generally necessary to obtain sufficient virus for protein expression. Recombinant virus are plaque purified and their DNA structure confirmed using standard methods well known in the art.

The rHuAFP cDNA fragment was isolated from plasmid pI18 with EcoRI/BamHI and purified using Geneclean as described *infra*. The cloning and expression of the HuAFP cDNA in baculovirus was performed using plasmid pVT-PlacZ. Human AFP cDNA was cloned into plasmid pVT-PlacZ (Fig. 7) in frame with the 3'-end of the insect derived melittin signal peptide sequence. The baculovirus vector pVT-PlacZ was modified by replacing the multiple cloning site with the oligonucleotide 5'-GATCTAGAATTCCGGATCCGGT-3' (SEQ ID NO: 21) and its complementary fragment, containing EcoRI and BamHI restriction sites in the 5' and 3' direction, reducing the number of non-AFP coding nucleotides between the melittin signal peptide cleavage site and the location of the AFP cDNA insert at the EcoRI

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endonuclease sequence. The insert was then ligated into the modified pVT-PLacZ vectors at the EcoRI and BamHI DNA sequences.

The generation of recombinant baculovirus containing rHuAFP coding sequences was performed according to standard techniques. Accordingly, purified recombinant baculovirus containing the coding sequences of HuAFP were generated by co-transfection of the pVT-PLacZ transfer vectors and wild-type baculovirus, followed by two rounds of plaque purification. Sf9 insect cells seeded at a density of  $1 \times 10^6$  cells/ml in 500 ml spinner flasks were infected with recombinant baculovirus in serum-free Grace medium at a multiplicity of infection of 5. The supernatant containing secreted rHuAFP was harvested and cells were removed by centrifuging at  $200 \times g$ . The rHuAFP containing medium was concentrated 10-20 fold by ultrafiltration with a YM30 Amicon membrane, dialysed overnight against PBS and then applied to a Con A lectin column (Pharmacia). Bound rHuAFP was eluted with 0.4M methyl  $\alpha$ -D mannopyranoside and was purified by elution from Mono Q resin during a linear gradient from 0-100% 1M NaCl in 20 mM phosphate buffer, pH 8.0. Recombinant HuAFP was characterized according to methods well known in the art.

I found that baculovirus produced rHuAFP represented approximately 20% of the total proteins secreted into serum-free medium by the Sf9 insect cells. This AFP was also found to be monomeric as analyzed by non-reducing alkaline PAGE. The majority of the baculovirus-derived HuAFP bound to immobilized Con A. This resulted in effective removal of more than 90% of contaminating proteins which were nonadhered to lectin columns. Final purification of the baculovirus derived rAFP preparations was accomplished by eluting protein with 270-310 mM NaCl from MonoQ beads, yielding a single

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polypeptide with an apparent molecular mass of approximately 68 kD. We obtained at least 1mg of purified protein was obtained per liter of growth culture.

5           The baculovirus-derived rHuAFP molecular weight is similar to the natural human molecule (Fig. 4B). This finding, in addition to the binding of baculovirus-derived rHuAFP and the observed non-adherence of E. coli-  
10           derived rHuAFP to the ConA column, indicates that the baculovirus-derived rHuAFP is glycosylated. However, the degree of glycosylation of the BrAFP is expected to be less than that of the native molecule, since Sf9 cells infected with recombinant baculovirus have been  
15           documented to be deficient in their ability to carry out complex glycosylation normally observed with higher eukaryotic derived proteins. Purity of the isolated baculovirus-derived rHuAFP was verified as a single band on APAGE and SDS-PAGE (Figs. 4A & 4B), and as a sole peak on FPLC and HPLC chromatograms as shown in Figures 4C and  
20           4D, respectively. N-terminal sequencing further confirmed the identity of pure rHuAFP. The N-terminal sequence of the rHuAFP was as follows: Asp-Leu-Glu-Phe-Met-Thr-Leu-His-Arg-Asn (SEQ ID NO: 22). Western blot analysis of serum free supernatants from recombinant  
25           baculovirus infected Sf9 cells detected a single immunoreactive band with monospecific anti-HuAFP Ab that was absent form the supernatant of uninfected or wild-type virus-infected Sf9 cells.

          Experiments were performed to evaluate the  
30           biological activity of the baculovirus produced HuAFP according to methods known in the art. For example, the immunosuppressive activity of 100 µg/ml of baculovirus-produced HuAFP was assessed by its ability to suppress human AMLR as described above. As shown in Figs. 5 and  
35           6A, baculovirus-derived rHuAFP inhibited the

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proliferative response of autoreactive lymphocytes stimulated by autologous non-T cells at 144 hours. The addition of an identical amount of human serum albumin failed to diminish lymphoproliferative responses.

5 To demonstrate that rHuAFP was the substrate responsible for the inhibition of autoproliiferating T cells, blocking of the rHuAFP-mediated suppression of the AMLR was performed using commercial murine anti-human AFP monoclonal antibodies (MAB). As shown in Fig. 5,  
10 suppression of proliferating autoreactive T cells by 100  $\mu\text{g/ml}$  *E. coli*- and baculovirus-derived rHuAFP was completely blocked by anti-HuAFP MAB. The addition of 100  $\mu\text{g/ml}$  of HSA did not diminish the AMLR response and the presence of MAB alone in the reaction culture was  
15 without any effect.

In addition, we tested the biological activity of rHuAFP to suppress the mitogen induced proliferation of peripheral blood lymphocytes (PBL) in RPMI tissue culture media supplemented only with 2 mg/ml purified human  
20 albumin (ICN, Mississauga, ON). As shown in Table II (below) and Fig. 6B, suppression of ConA stimulated PBLs occurred with 100  $\mu\text{g/ml}$  rHuAFP whereas the same concentration of albumin was ineffective.

Other rHuAFPs (e.g., rHuAFP (Amino acids 1(Thr) -  
25 590 (Val); SEQ ID NO: 5); Domain I (Amino acids 1 (Thr) - 197 (Ser), SEQ ID NO: 6; Domain II (Amino acids 198 (Ser) - 389 (Ser), SEQ ID NO: 7); Domain III (Amino acids 390 (Gln) - 590 (Val), SEQ ID NO: 8); Domain I + II (Amino acids 1 (Thr) - 389 (Ser), SEQ ID NO: 9); Domain II +  
30 III, (Amino acids 198 (Ser) - 590 (Val), SEQ ID NO: 10); rHuAFP Fragment I (Amino acids 266 (Met) - 590 (Val), SEQ ID NO: 11) can be produced using the above described...  
baculovirus expression system according to standard methods, e.g., any of the methods described herein.

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In one working example, the vector pVT-PLacZ/HuAFP (amino acids 1 - 590) is constructed by inserting the cDNA for HuAFP into pVT-P10, an intermediate vector in the construction of pVT-PLacZ (Richardson et al., (1992)

5 Engineering Glycoproteins for secretion using the baculovirus expression system. In: *Baculovirus and Recombinant Protein Production Processes*, eds., J.M. Viak, E.-J. Schlaeger, and A.R. Bernard, Editiones Roche, Basel, Switzerland. pp. 67-73.). The pVT-P10 vector is

10 digested with BamHI, followed by incubation with mung bean Nuclease (New England Biolabs, Mississauga, Ont.). The vector is then further hydrolyzed with EcoRI downstream of the blunt-end BamHI site to facilitate directional cloning of the HuAFP cDNA. The rHuAFP cDNA

15 encoding amino acids 1-590 is obtained by PCR amplification, employing the following oligonucleotide primers: (5'-AAAAAACTCGAGATACACTGCATAGAAATGAA-3'; SEQ ID NO: 23), containing an XhoI site and (5'-

20 AAAAAGAATTCTTAACTCCCAAAGCAGCACG-3'; SEQ ID NO:24), containing an EcoRI site, and plasmid p118 as the template DNA containing the coding region of HuAFP. The PCR reaction is performed according to standard methods, e.g., in a reaction mixture containing 34  $\mu$ L H<sub>2</sub>O, 10 $\mu$ L 10 x reaction buffer, 20  $\mu$ L dNTP, 2 $\mu$ L DNA template, 10  $\mu$ L 10

25 pmol/ $\mu$ L 5' primer, 10  $\mu$ L 10 pmol/ $\mu$ L 3' primer, 1  $\mu$ L glycerol, 10  $\mu$ L DMSO, and 1  $\mu$ L Pfu polymerase. Annealing, extension, and denaturation temperatures are also performed according to standard conditions, e.g., 50°C, 72°C, and 94°C, respectively, for 30 cycles, using

30 the GeneAmp PCR System 9600 (Perking Elmer Cetus). DNA from the PCR reaction is purified using the Genaclean kit (Bio 11 Inc., LaJolla, CA). The rHuAFP cDNA fragment is first digested with XhoI followed by treatment with mung bean nuclease. Next, the HuAFP cDNA is digested with

35 EcoRI to facilitate directional cloning into pVT-p10.

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The PCR-produced rHuAFP cDNA is ligated into the blunt ended 5' BamH1 site and into the 3' EcoR1 site. The  $\beta$ -galactosidase gene containing at the 3' end a polyadenylation site from SV40, isolated from the vector pJV-Nhe1 (Vialard et al., (1990) Synthesis of the membrane fusion and hemagglutinin proteins of Measles Virus, using a novel baculovirus vector containing the  $\beta$ -galactosidase gene, J. Virology 64: 37-50) by using the restriction enzyme BamH1, is then inserted into the compatible BgIII, producing the final construct: pVT-PLacZ/HuAFP (containing amino acids 1-590). Such a construct is then used for expressing rHuAFP (amino acids 1-590).

#### Fragments and Analogs

The invention includes biologically active fragments of rHuAFP. A biologically active fragment of rHuAFP is one that possesses at least one of the following activities: (a) directs a specific interaction with a target cell, e.g., binds to a cell expressing a receptor which is recognized by rHuAFP (e.g., the membrane of a cancer cell such as an MCF-7 or a bone marrow cell); (b) halts, reduces, or inhibits the growth of a neoplasm or an autoreactive immune cell (e.g., binds to a cell surface receptor and imparts an anti-proliferative signal); stimulates, increases, expands, or otherwise causes the proliferation of a cell such as a bone marrow cell (e.g., binds to a cell surface receptor a proliferative or stimulating-signal); or blocks or inhibits or prevents an immunopathologic antibody reaction. The ability of rHuAFP fragments or analogs to bind to a receptor which is recognized rHuAFP can be tested using any standard binding assay known in the art. Biological activity of such fragments and analogs are tested according to methods known in the art (e.g., those described herein).

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In general, fragments of rHuAFP are produced according to the techniques of polypeptide expression and purification described supra. For example, suitable fragments of rHuAFP can be produced by transformation of a suitable host bacterial cell with part of an HuAFP-encoding cDNA fragment (e.g., the cDNA described above) in a suitable expression vehicle. Alternatively, such fragments can be generated by standard techniques of PCR and cloned into the expression vectors (supra).

10 Fragments can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984, The Pierce Chemical Co., Rockford, Il). Accordingly, once a fragment of rHuAFP is expressed, it may be isolated by various chromatographic

15 and/or immunological methods known in the art. Lysis and fractionation of rHuAFP-containing cells prior to affinity chromatography may be performed by standard methods. The ability of a candidate rHuAFP fragment to exhibit a biological activity of alpha-fetoprotein is

20 assessed by methods known to those skilled in the art (e.g., those described herein).

As is discussed above, a rHuAFP fragment may also be expressed as a fusion protein with maltose binding protein produced in E. coli. Using the maltose binding

25 protein fusion and purification system (New England Biolabs, Beverly, MA), the cloned human cDNA sequence can be inserted downstream and in frame of the gene encoding maltose binding protein (malE), and the malE fusion protein can then be overexpressed. In the absence of

30 convenient restriction sites in the human cDNA sequence, PCR can be used to introduce restriction sites compatible with the vector at the 5' and 3' end of the cDNA fragment to facilitate insertion of the cDNA fragment into the vector. Following expression of the fusion protein,

35 it can be purified by affinity chromatography. For



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example, the fusion protein can be purified by virtue of the ability of the maltose binding protein portion of the fusion protein to bind to amylose immobilized on a column.

5           To facilitate protein purification, the pMalE plasmid contains a factor Xa cleavage site upstream of the site into which the cDNA is inserted into the vector. Thus, the fusion protein purified as described above can then be cleaved with factor Xa to separate the maltose  
10 binding protein from a fragment of the recombinant human cDNA gene product. The cleavage products can be subjected to further chromatography to purify rHuAFP from the maltose binding protein. Alternatively, a fragment of rHuAFP may be expressed as a fusion protein containing  
15 a polyhistidine tag can be produced. Such an alpha-fetoprotein fusion protein may then be isolated by binding of the polyhistidine tag to an affinity column having a nickel moiety which binds the polyhistidine region with high affinity. The fusion protein may then  
20 be eluted by shifting the pH within the affinity column. The rHuAFP can be released from the polyhistidine sequences present in the resultant fusion protein by cleavage of the fusion protein with specific proteases.

Recombinant HuAFP fragment expression products  
25 (e.g., produced by any of the prokaryotic systems described supra) may be assayed by immunological procedures, such as Western blot, immunoprecipitation analysis of recombinant cell extracts, or immunofluorescence (using, e.g., the methods described in  
30 Ausubel et al., *Current Protocols In Molecular Biology*, Greene Publishing Associates and Wiley Interscience (John Wiley & Sons), New York, 1994).

If desired, the purified recombinant gene product or fragment thereof can then be used to raise polyclonal  
35 or monoclonal antibodies against the human recombinant

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alpha-fetoprotein using well-known methods (see Coligan et al., eds., *Current Protocols in Immunology*, 1992, Greene Publishing Associates and Wiley-Interscience). To generate monoclonal antibodies, a mouse can be immunized  
5 with the recombinant protein, and antibody-secreting B cells isolated and immortalized with a non-secretory myeloma cell fusion partner. Hybridomas are then screened for production of recombinant human alpha-fetoprotein (or a fragment or analog thereof)-specific  
10 antibodies and cloned to obtain a homogenous cell population which produces monoclonal antibodies.

As used herein, the term "fragment," as applied to a rHuAFP polypeptide, is preferably at least 20 contiguous amino acids, preferably at least 50 contiguous  
15 amino acids, more preferably at least 100 contiguous amino acids, and most preferably at least 200 to 400 or more contiguous amino acids in length. Fragments of rHuAFP molecules can be generated by methods known to those skilled in the art, e.g., proteolytic cleavage or  
20 expression of recombinant peptides, or may result from normal protein processing (e.g., removal of amino acids from nascent polypeptide that are not required for biological activity).

Recombinant HuAFP fragments of interest include,  
25 but are not limited to, Domain I (amino acids 1 (Thr) - 197 (Ser), see Fig. 1, SEQ ID NO: 6), Domain II (amino acids 198 (Ser) - 389 (Ser), see Fig. 1, SEQ ID NO: 7), Domain III (amino acids 390 (Gln) - 590 (Val), see Fig. 1, SEQ ID NO:   ), Domain I+II (amino acids 1 (Thr) - 389  
30 (Ser), see Fig. 1, SEQ ID NO: 9), Domain II+III (amino acids 198 (Ser) - 590 (Val), see Fig. 1, SEQ ID NO: 10), and rHuAFP Fragment I (amino acids 266 (Met) - 590 (Val), see Fig. 1, SEQ ID NO: 11). Activity of a fragment is evaluated experimentally using conventional techniques  
35 and assays, e.g., the assays described herein.

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The invention further includes analogs of full-length rHuAFP or fragments thereof. Analogs can differ from rHuAFP by amino acid sequence differences, or by modifications (e.g., post-translational modifications) which do not affect sequence, or by both. Analogs of the invention will generally exhibit at least 80%, more preferably 85%, and most preferably 90% or even 99% amino acid identity with all or part of a rHuAFP amino acid sequence. Modifications (which do not normally alter primary sequence) include in vivo, or in vitro chemical derivatization of polypeptides, e.g., acetylation, or carboxylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes.

Analogs can also differ from the naturally occurring rHuAFP by alterations in primary sequence, for example, substitution of one amino acid for another with similar characteristics (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the polypeptide's biological activity. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, 1989, or Ausubel et al., supra)). Also included are cyclized peptide molecules and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g.,  $\beta$  or  $\gamma$  amino acids, or L-amino acids with non-natural side chains (see e.g., Noren et al., *Science* 244:182, 1989). Methods for site-specific incorporation of non-natural amino acids into the protein backbone of proteins is described, e.g., in Ellman et al., *Science* 255:197, 1992.

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Also included are chemically synthesized polypeptides or peptides with modified peptide bonds (e.g., non-peptide bonds as described in U.S. Pat. No. 4,897,445 and U.S. Pat. No. 5,059,653) or modified side chains to obtain the  
5 desired pharmaceutical properties as described herein. Useful mutants and analogs are identified using conventional methods, e.g., those described herein.

#### Recombinant HuAFP As An Immunosuppressive Agent

Immunosuppressive attributes of rHuAFP (or a  
10 fragment or analog thereof) are evaluated by any standard assay for analysis of immunoregulatory activity in vivo or in vitro. As discussed infra, the art provides a number of animal systems for in vivo testing of immunosuppressive characteristics of rHuAFP (or a  
15 fragment or analog thereof) on an autoimmune disease, e.g., the nonobese diabetic (NOD) mouse. Furthermore, a wide variety of in vitro systems are also available for testing immunosuppressive aspects of rHuAFP e.g., one such in vitro assay evaluates the inhibition of  
20 autoantigen-induced proliferation of T cells in an autologous mixed lymphocyte reaction (AMLR).

The following examples demonstrate that unglycosylated rHuAFP and a fragment of rHuAFP inhibit T cell autoprolieration in response to self antigens.  
25 These examples are provided to illustrate, not limit, the invention.

### EXPERIMENTAL

#### MATERIALS AND METHODS

##### Gel Electrophoresis, Immunoblotting and 30 Purification

The purity and characterization of rHuAFP was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and nondenaturing alkaline PAGE (APAGE) according to standard methods. Gels were  
35 subsequently analyzed either by staining with Coomassie

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brilliant blue or by transferring electrophoretically separated polypeptides to Immobilon PVDF membranes (Millipore, Mississauga, ON) for immunoblotting analysis. Recombinant HuAFP-monospecific rabbit anti-natural HuAFP polyclonal antibody complexes were identified by alkaline-phosphatase-conjugated goat anti-rabbit IgG and the immunoreactive bands were detected with the BCIP/NBT color development solution (Bio-Rad Laboratories, Mississauga, ON) according to the manufacturer's instructions.

Column chromatography was performed according to standard methods.

#### Polymerase Chain Reaction (PCR) rHuAFP Fragments

Plasmid constructs encoding fragments of human alpha-fetoprotein were prepared using polymerase chain reaction (PCR) techniques known to those skilled in the art of molecular biology, using oligonucleotide primers designed to amplify specific portions of the human alpha-fetoprotein gene (see e.g., *PCR Technology*, H.A. Erlich, ed., Stockton Press, New York, 1989; *PCR Protocols: A Guide to Methods and Applications*, M.A. Innis, David H. Gelfand, John J. Sninsky, and Thomas J. White, eds., Academic Press, Inc., New York, 1990, and Ausubel et. al., supra).

The following six rHuAFP fragments were prepared to evaluate their biological activity (e.g., according to the -methods disclosed herein):

Domain I	Amino acids 1 (Thr) - 197 (Ser),	(Fig. 1, SEQ ID NO: 6)
Domain II	Amino acids 198 (Ser) - 389 (Ser),	(Fig. 1, SEQ ID NO: 7)
Domain III	Amino acids 390 (Gln) - 590 (Val),	(Fig. 1, SEQ ID NO: 8)
Domain I + II	Amino acids 1 (Thr) - 389 (Ser),	(Fig. 1, SEQ ID NO: 9)
Domain II + III	Amino acids 198 (Ser) - 590 (Val),	(Fig. 1, SEQ ID NO: 10)
rHuAFP Fragment I	Amino acids 266 (Met) - 590 (Val),	(Fig. 1, SEQ ID NO: 11)

Amino acids sequences were deduced from those shown for human alpha-fetoprotein (1(Thr) - 590 (Val); SEQ ID NO: 5

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in Fig. 1. Fragments of rHuAFP designated Domain I, Domain II, Domain III, Domain I+II, Domain II+III and rHuAFP Fragment I were synthesized using standard PCR reaction conditions in 100  $\mu$ L reactions containing 34  $\mu$ L H<sub>2</sub>O, 10  $\mu$ L 10X reaction buffer, 20  $\mu$ L 1 mM dNTP, 2  $\mu$ L DNA template (HuAFP cloned in pI18), appropriate 5' and 3' oligonucleotide primers (10  $\mu$ L 10 pmol/ $\mu$ L 5' primer, 10  $\mu$ L 10 pmol/ $\mu$ L 3' primer), 1  $\mu$ L glycerol, 10  $\mu$ L DMSO, and 1  $\mu$ L Pfu polymerase (Stratagene, LaJolla, CA). Primers used for PCR amplifications were:

DomI25	5'-AAAAAAGGTACACACTGCATAGAAATGAA-3'	(SEQ ID NO: 14)
DomI3	5'-AAAAAAGGATCCTTAGCTTTCTCTTAATTCTTT-3'	(SEQ ID NO: 15)
DomII5	5'-AAAAAATCGATATGAGCTTGTTAAATCAACAT-3'	(SEQ ID NO: 16)
DomII3	5'-AAAAAAGGATCCTTAGCTCTCCTGGATGTATTT-3'	(SEQ ID NO: 17)
15 DomIII5	5'-AAAAAATCGATATGCAAGCATTGGCAAAGCGA-3'	(SEQ ID NO: 18)
DomIII3	5'-AAAAAAGGATCCTTAACTCCCAAAGCAGCAG-3'	(SEQ ID NO: 19)
5'rHuAFP Fragment I	5'-AAAAAATCGATATGCTCATATGTCTCTCAA-3'	(SEQ ID NO: 20)

Accordingly, primer pairs DomI25 and DomI3, DomII5 and DomII3, DomIII5 and DomIII3, 5'rHuAFP Fragment I and DomIII3, DomI25 and DomII3, and DomII5 and DomIII3 were used to isolate cDNA sequences of Domain I, Domain II, Domain III, rHuAFP Fragment I, Domain I+II, and Domain II+III, respectively, of rHuAFP. Annealing, extension, and denaturation temperatures were 50°C, 72°C, and 94°C, respectively, for 30 cycles. PCR products were purified according to standard methods. Purified PCR products encoding Domain I and Domain I+II were digested individually with KpnI and BamHI and cloned separately into KpnI/BamHI- treated pTrp4. Purified PCR products encoding Domain II, Domain III, Domain II+III, and rHuAFP Fragment I were digested individually with Bsp106I and BamHI and were cloned separately into Bsp106I/BamHI- treated pTrp4. Each plasmid construct was subsequently transformed into competent *E. coli* cells. Since the expression product will begin with the amino acid

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sequence encoded by the translation start signal methionine, it is expected that such signal will be removed, or in any event, not affect the bioactivity of the ultimate expression product.

5           Autologous Mixed Lymphocyte Reactions (AMLR)

Isolation of human peripheral blood mononuclear cells (PBMC), their fractionation into non-T cell populations, and the AMLR, were performed according to standard procedures. Responder T cells were isolated by  
10 passing  $1.5 \times 10^8$  PMBC over a commercial Ig-anti-Ig affinity column (Biotek Laboratories) and  $2 \times 10^5$  responder cells were subsequently cultured with  $2 \times 10^5$  autologous  $^{137}\text{Cs}$ -irradiated (2500 rads) non-T stimulator cells from a single donor. The medium employed consisted  
15 of RPMI-1640 supplemented with 20 mM HEPES (Gibco),  $5 \times 10^{-5}$  M 2-mercaptoethanol (BDH, Montreal, QC), 4 mM L-glutamine (Gibco), 100 U/ml penicillin (Gibco) and 100  $\mu\text{g/ml}$  streptomycin sulfate, with the addition of 10% fresh human serum autologous to the responder T cell  
20 donor for the AMLR. Varying concentrations of purified rHuAFP, human serum albumin (HSA), anti-HuAFP monoclonal antibodies clone #164 (125  $\mu\text{g/ml}$  final concentration in culture) (Leinco Technologies, St. Louis, MO) were added at the initiation of cultures. AMLR cultures were  
25 incubated for 4 to 7 days, at  $37^\circ\text{C}$  in 95% air and 5%  $\text{CO}_2$ . At the indicated intervals, DNA synthesis was assayed by a 6 hour pulse with 1  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine (specific activity 56 to 80 Ci/mmmole, ICN). The cultures were harvested on a multiple sample harvester (Skatron,  
30 Sterling, VA), and the incorporation of  $^3\text{H}$ -TdR was measured in a Packard 2500 TR liquid scintillation counter. Results are expressed as mean cpm  $\pm$  the standard error of the mean of triplicate or quadruplicate cultures.

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### Peripheral Blood Lymphocyte (PBL) Assays

Heparinized blood from normal donors was diluted 1:1 with PBS and human peripheral blood lymphocytes (PBL) were separated from red blood cells by density centrifugation on Ficoll-Hypaque (Sigma, St. Louis, MO). They were washed at least 3 times with PBS and verified for cell viability by the Trypan Blue exclusion method. Human PBL ( $2.5 \times 10^5$  cells) were cultured according to standard methods. Results are expressed as the mean cpm thymidine incorporation  $\pm$  SEM of triplicate cultures.

### RESULTS

#### Expression and Purification

Purity of isolated rHuAFP expressed in *E. coli* was verified as a single band on Coomassie stained APAGE and SDS-PAGE are shown in Fig. 1A-1B, respectively. Soluble monomeric rHuAFP derived from *E. coli* was obtained by eluting a protein fraction containing rHuAFP employing Q-sepharose chromatography. Approximately 1 mg of pure rHuAFP per liter of bacterial culture was recovered as a single homogeneous peak by FPLC Mono-Q anion exchange with 220-230 mM NaCl and migrated at approximately 65 kD on SDS-PAGE (Fig. 1B). Recombinant HuAFP exhibits a lower molecular weight on SDS-PAGE than natural HuAFP, since prokaryotic expression systems lack the enzymatic machinery required for glycosylation of proteins. Rechromatographed samples of pure rHuAFP on FPLC and HPLC yielded a single peak as shown in Fig. 1C and Fig. 1D, confirming the purity of the rHuAFP preparation. In addition, N-terminal sequencing data correspond to the expected amino acid sequence at the N-terminus of rHuAFP.

In addition, *E. coli* containing the expression plasmid encoding rHuAFP were cultured as described above. Fig. 2 (lane D) shows the SDS-PAGE profile of purified rHuAFP Fragment I. N-terminal amino acid sequence analysis showed that rHuAFP Fragment I possessed the



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amino acid sequence Ser<sub>267</sub>-Tyr-Ile-Cys-Ser-Gln-Gln-Asp-Thr<sub>275</sub> (SEQ ID NO: 13) which corresponds to the expected N-terminal amino acid sequence of rHuAFP Fragment I (see Fig. 1, SEQ ID NO: 11) where the initiating methionine is 5 cleaved intracellularly.

Inhibition of the Autologous Mixed Lymphocyte Reactions (AMLR)

The immunosuppressive activity of rHuAFP was assessed by its ability to suppress human autologous 10 mixed lymphocyte reactions (AMLR). As shown in Fig. 8A, rHuAFP inhibited the proliferative response of autoreactive lymphocytes stimulated by autologous non-T cells, throughout the 4 to 7 day time course measuring autoproliiferation. Results from dose-response studies 15 performed at the peak of T cell autoproliiferation, as shown in Fig. 8B, demonstrate that the addition of rHuAFP at the initiation of cultures suppressed the AMLR in a dose-dependent manner. Furthermore, parallel viability studies established that the inhibitory activity of 20 rHuAFP on human autoreactive T cells was not due to non-specific cytotoxic effects.

To further substantiate that rHuAFP was the agent responsible for the inhibition of autoproliiferating T cells, blocking of rHuAFP-mediated suppression of the 25 AMLR was performed using commercial murine anti-human AFP monoclonal antibodies (MAb). As illustrated in Fig. 5, suppression of proliferating autoreactive T cells by 100 µg/ml of rHuAFP was completely blocked by anti-HuAFP MAb. The addition of 100 µg/ml of HSA did not diminish the 30 AMLR response and the presence of MAb alone in the reaction culture was without any effect.

Recombinant polypeptides produced in prokaryotic expression systems are at risk for contamination with host cell lipopolysaccharide (LPS) during their isolation 35 from bacteria. It has been demonstrated that small

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amounts of LPS can antagonize the biological activities of cytokines, thereby impairing the immune responsiveness of macrophages. Accordingly, the effect of endotoxin on various rHuAFP preparations was evaluated by performing 5 AMLR experiments with recombinant protein depleted of endotoxin by passage over Detoxi-gel (Pierce) versus that of rHuAFP which was untreated. Results of these experiments showed that both preparations had equivalent levels of immunosuppressive activity.

10 As shown in Figure 8A and Figure 8B, the results of this study also demonstrate that rHuAFP suppresses the proliferation of autoreaction T cells with a potency equivalent to glycosylated nHuAFP by eliciting inhibitory effects on autoproliiferating T cells throughout the in 15 vitro reactions, with highly significant inhibition being achieved with rHuAFP concentrations ranging from 5  $\mu$ g/ml to 100  $\mu$ g/ml. In addition, as shown in Table I and Fig. 3, rHuAFP Fragment I inhibited the proliferative response of autoreactive lymphocytes stimulated by autologous non- 20 T cells at 144 hours. Moreover, as shown in Fig. 3, Domains I and III also inhibited the AMLR.

Table I

Reaction Setup	Thymidine Incorporation (CPM)
T Cells	7118 $\pm$ 964
25 AMLR	83103 $\pm$ 6480
AMLR + rHuAFP Fragment I (100 $\mu$ g/ml)	29692 $\pm$ 2963

Inhibition of the Peripheral Blood Lymphocyte  
Reactions (PBL)

30 The immunosuppressive activity of 100  $\mu$ g/ml rHuAFP Fragment I, and E. coli- and baculovirus-derived rHuAFP (described below) was also assessed for the ability to

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suppress human peripheral blood lymphocyte reactions (PBL). As shown in Table II, E. coli- and baculovirus-derived rHuAFP and Fragment I (produced as described above) were found to inhibit the proliferative responses of Con A stimulated human peripheral blood lymphocytes.

Table II

3H-Thymidine Incorporation (CPM $\pm$ SE)			
No protein	102,353 $\pm$ 5,566	91,502 $\pm$ 4,333	99,700 $\pm$ 4,464
100 $\mu$ g/ml Human Albumin	89,860 $\pm$ 5,800	82,924 $\pm$ 11,085	94,123 $\pm$ 1,633
100 $\mu$ g/ml rHuAFP- <u>E. coli</u>	33,641 $\pm$ 3,893	-	-
100 $\mu$ g/ml rHuAFP-Baculovirus	-	31,331 $\pm$ 6,303	-
100 $\mu$ g/ml HuAFP Fragment I	-	-	39,019 $\pm$ 161

### Autoimmune Disease

As is discussed above, autoimmune diseases are characterized by a loss of tolerance to self antigens, causing cells of the immune systems, e.g., T or B cells (or both), to react against self tissue antigens. Autoimmune diseases may involve any organ system, although some are affected more commonly than others. Examples of tissues affected by autoimmune conditions include: the white matter of the brain and spinal cord in multiple sclerosis; the lining of the joints in rheumatoid arthritis; and the insulin secreting  $\beta$  islet cells of the pancreas in insulin-dependent diabetes mellitus. Other forms of autoimmune disease destroy the connections between nerve and muscle in myasthenia gravis or destroy the kidneys and other organs in systemic lupus erythematosus. Examples of other autoimmune diseases include, without limitation, Addison's disease, Crohn's disease, Graves' disease, psoriasis, scleroderma, and ulcerative colitis.

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The art provides a wide variety of experimental animal systems, transgenic and non-transgenic, for testing therapies for human illness involving autoimmune diseases (see e.g., Paul, W.E., *Fundamental Immunology*, 2nd ed., Raven Press, New York, 1989; and Kandel et al. *Principles of Neural Science*, 3rd ed., Appleton and Lange, Norwalk, CT, 1991; and *Current Protocols In Immunology*, Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., and Strober, eds., Green Publishing Associates (John Wiley & Sons), New York, 1992). Based on the above-described experimental results showing immunosuppressive activity of unglycosylated rHuAFP, it is reasonable to believe that other autoimmune diseases can be treated by administration of such rHuAFP (or a fragment or analog thereof) produced in a prokaryotic system. Accordingly, the invention provides the use of rHuAFP (or a fragment or analog thereof) for treatment (i.e., prevention or suppression or amelioration or promotion of remission) of any autoimmune disease.

There now follow examples of animal systems useful for evaluating the efficacy of recombinant human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof in treating autoimmune diseases. These examples are provided for the purpose of illustrating, not limiting, the invention.

#### Multiple Sclerosis

Multiple sclerosis (MS) is a demyelinating disease involving scattered areas of the white matter of the central nervous system. In MS, myelin basic protein and proteolipid protein are the major targets of an autoimmune response involving T lymphocytes, among other immune system components. Loss of the myelin sheath of nerve cells (demyelination) occurs, resulting in neurological symptoms that culminate in coma or paralysis.

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Experimental autoimmune encephalomyelitis (EAE) is a primary model used in the art to examine and assess the effectiveness of therapeutic agents for treating MS. EAE is an inflammatory autoimmune demyelinating disease induced in laboratory animals by immunization with central nervous system tissue. When animals (e.g., mice, rats, guinea pigs, rabbits, monkeys, etc.) are injected with adjuvant, e.g., complete Freund's adjuvant, plus myelin basic protein or proteolipid protein, EAE is induced, which is similar, pathologically to MS (see e.g., Alvord et al., *Experimental Allergic Encephalomyelitis-A Useful Model for Multiple Sclerosis*, Liss, New York, 1984; Swanborg, *Meth. Enzymol.* 162:413, 1988; and McCarron et al., *J. Immunol.*, 147: 3296, 1991.)

To evaluate rHuAFP or a fragment or analog thereof, EAE is induced in an appropriate laboratory animal, e.g., a mouse or rabbit, according to methods known in the art. To evaluate the compound's immunosuppressive effect on EAE, i.e., its ability to prevent or ameliorate EAE, the compound is administered according to standard methods, e.g., intravenously or intraperitoneal, at an appropriate dosage on a daily basis. Generally, administration is initiated prior to inducing EAE and/or after the clinical appearance of EAE. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test molecules on EAE is monitored according to any standard method. For example, weight loss and muscle paralysis in EAE-induced animals is monitored on a daily basis. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., *Current Protocols In Molecular Biology*, Greene Publishing Associates (John Wiley & Son), New York, 1994; Bancroft

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and Stevens, *Theory and Practice of Histochemical Techniques*, Churchill Livingstone, 1982) of brain and spinal cord tissues is performed and tissue samples examined microscopically for evidence of EAE, e.g.,  
5 evidence of perivascular cellular infiltrates. Comparative studies between treated and control animals are used to determine the relative efficacy of the test molecules in preventing or ameliorating EAE. A molecule which prevents or ameliorates (decreases or suppresses or  
10 relieves or promotes remission of) the symptoms of EAE is considered useful in the invention.

#### Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common chronic illness in which the synovial membrane of multiple joints  
15 becomes inflamed, causing damage to cartilage and bone. RA is associated with human lymphocyte antigen (HLA)-DR4 and considered to be an autoimmune disorder involving T cells, see e.g., Sewell et al., Lancet 341: 283, 1993. RA results from a complex interaction of synovial cells  
20 with various cellular elements (and their soluble products) that infiltrate from the circulation into the synovial lining of joints. A series of biological events occur which ultimately lead to a lesion which invades and erodes collagen and the cartilage matrix of the joint.

25 A number of animal models of RA, e.g., the MRL-*lpr/lpr* mouse, are known in the art which develop a form of arthritis resembling the human disease (see e.g., *Fundamental Immunology*, supra). Alternatively, autoimmune collagen arthritis (ACA) and adjuvant  
30 arthritis (AA) can be induced in an appropriate animal according to standard methods.

To evaluate rHuAFP or a fragment or analog thereof on immunosuppressive on RA, i.e., the compound's ability to prevent or ameliorate RA, the test molecule is  
35 administered to a MRL-*lpr/lpr* mouse according to standard

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methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration is initiated prior to the onset of RA and/or after the clinical appearance of RA. Control  
5 animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test molecule on RA is monitored according to standard methods. For example, analysis of the cellular component(s) of a synovial joint  
10 are monitored on a daily basis. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of the synovial joint is performed and tissue samples examined microscopically  
15 for evidence of RA, e.g., evidence of erosion of collagen and cartilage matrix in a joint. Comparative studies between treated and control animals are used to determine the relative efficacy of the test molecule in preventing or ameliorating RA. A test molecule which prevents or  
20 ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of RA is considered useful in the invention.

#### Myasthenia Gravis

Myasthenia gravis (MG) is a disorder of  
25 neuromuscular transmission in which there are autoantibodies against acetylcholine receptors of neuromuscular junctions. Antibodies attack the junction, causing weakness and paralysis. Females are afflicted twice as often as males, typically during the third  
30 decade of life. Muscular weakness is the predominant feature of the disease. Clinical signs include drooping of the eyelids and double vision. There is an association between MG and hyperthyroidism.

Experimental autoimmune MG (EAMG) has been studied  
35 in a variety of animals including rabbits, monkeys, Lewis

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rats and inbred strains of mice (see e.g., *Principles of Neural Science*, supra), the symptoms of EAMG resemble the essential characteristics of the human disease. A single injection of acetylcholine receptor, e.g., purified from  
5 the electric organs of the eel Torpedo californica, along with adjuvants, causes an acute phase of weakness within 8 to 12 days and then chronic weakness after about 30 days. The response to the eel receptor is T cell dependent. The C57BL/6 strain (H-2<sup>B</sup>) is a high responder  
10 to Torpedo receptor and highly susceptible to EAMG.

To evaluate rHuAFP or a fragment or analog thereof, EAMG is induced in an appropriate laboratory animal, e.g., the C57BL/6 strain (H-2<sup>B</sup>) mouse, according to methods known in the art. To evaluate the compound's  
15 immunosuppressive effect on EAMG, i.e., its ability to prevent or ameliorate EAMG, the compound is administered according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration is initiated prior to  
20 inducing EAMG and/or after the clinical appearance of EAMG. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test molecules on EAMG is monitored according to standard methods. For  
25 example, nerve stimulation in an electromyographic muscle assay (e.g., according to the methods of Pachner et al., Ann. Neurol. 11:48, 1982) in EAMG-induced animals can be assayed. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical  
30 procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of tissue samples is performed and tissue samples examined microscopically for evidence of EAMG, e.g., evidence of monocyte infiltration and/or autoantibody localization at acetylcholine receptors of  
35 neuromuscular junctions. Comparative studies between



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treated and control animals are used to determine the relative efficacy of the test molecules in preventing or ameliorating EAMG. A molecule which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of EAMG is considered useful in the invention.

#### Insulin-Dependent Diabetes Mellitus

Diabetes is a disorder of glucose metabolism.

Insulin-dependent diabetes mellitus (IDDM), also known as Type I diabetes, is an autoimmune disease characterized by T-cell mediated destruction of pancreatic  $\beta$  cells in the islets of Langerhans, accompanied by an immune response to a diversity of self peptides leading to hyperglycemia, among other pathological events. IDDM patients depend on exogenous insulin to maintain normal glucose metabolism. Humans at risk for developing IDDM can be identified prior to onset of hyperglycemia by the abnormal occurrence of autoantibodies to insulin, islet cells, glutamic acid carboxylase, as well as other autologous proteins (see e.g., Baekkeskov et al., J. Clin. Invest. 79:926, 1987; Dean et al., Diabetologia 29:339, 1986; Rossini et al., Annu. Rev. Immunol. 3:289, 1985; Srikanta et al., N. Engl. J. Med. 308:322, 1983). Autoantibody patterns, in general, are predictive for the eventual disease progression and/or risk for developing the disease (see e.g., Keller et al., Lancet 341:927, 1993).

Examples of animal models which spontaneously develop IDDM resembling the human disease include the Bio-Breeding (BB) rat and nonobese diabetic (NOD) mouse. Diabetes is also experimentally induced by streptozotocin.

The BB rat spontaneously develops a disease similar to IDDM, with insulinitis (infiltration of mononuclear cells into the pancreatic islets) and

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autoantibodies against self cells and insulin (see e.g., Baekkeskov et al., J. Clin. Invest. 79:926, 1987; Rossini et al, supra; Nakhooda et al., Diabetes 26: 100, 1977; Dean et al., Clin. Exp. Immunol. 69: 308, 1987).

5 NOD mice typically develop insulinitis between 5 and 8 weeks of age, and by 7 months 70% of the females and 40% of the males become diabetic. T cells transferred from diabetic mice to young nondiabetic NOD mice induce diabetes within 2 to 3 weeks (see e.g., Bendelac et al.,  
10 J. Exp. Med. 166:823, 1987). NOD mice usually die within 1 to 2 months after the onset of diabetes unless they receive insulin therapy.

Chemically induced diabetes is accomplished using multiple injections of small doses of streptozotocin, a  
15 drug toxic for pancreatic  $\beta$  cells, which causes severe insulinitis and diabetes (see e.g., Kikutani et al., Adv. Immunol. 51:285, 1992).

Accordingly, the art provides a variety animal models resembling human IDDM which can be used to examine  
20 and assess approaches for the prevention or amelioration of diabetes involving rHuAFP (or a fragment or analog thereof).

To evaluate the immunosuppressive effect of rHuAFP or a fragment or analog thereof on the development of  
25 diabetes mouse, i.e., the compound's ability to treat or prevent insulinitis and diabetes, the test compound is administered to an appropriate test animal, e.g., a NOD mouse, according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily  
30 basis. Generally, administration is initiated prior to the onset of insulinitis and diabetes and/or after the clinical appearance of diabetic characteristics. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related  
35 molecules. The effect of test molecules on insulinitis and

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diabetes is monitored according to standard methods. For example, weight loss, ketone body formation, and blood glucose concentration is monitored on a daily basis. If desired, histological inspection (e.g., by using any  
5 standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of pancreatic islet cells is performed and tissue samples examined microscopically for evidence of insulinitis and  $\beta$  cell destruction. Comparative studies  
10 between treated and control animals are used to determine the relative efficacy of the test molecules in preventing or ameliorating the diabetic condition. A molecule which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of  
15 diabetes, e.g., IDDM, is considered useful in the invention.

#### Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a severe systemic autoimmune disease. About 90% of patients with  
20 this disease are young women. This marked preponderance of females is not seen before puberty or after menopause. The illness generally begins in young adulthood when a characteristic skin rash appears over cheekbones and forehead. Hair loss is common, as is severe kidney  
25 damage, arthritis, accumulation of fluid around the heart and inflammation of the lining of the lungs. In nearly half of the patients the blood vessels of the brain also become inflamed, leading to paralysis and convulsions. The activity of the disease, like other autoimmune  
30 diseases, can fluctuate: long quiescent periods of good health can terminate abruptly and inexplicably with the onset of a new attack. A large number of different autoantibodies are known to occur in SLE, e.g., autoantibodies against DNA, RNA and histones (see, e.g.,  
35 Fundamental Immunology, supra)

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A number of animal models of human SLE, e.g., inbred mouse strains including NZB mice and their F<sub>1</sub> hybrids, MRL mice, and BXSB mice, are known in the art (see e.g., Bielschowsky et al. Proc. Univ. Otago Med. Sch. 37:9, 1959; Braverman et al., J. Invest. Derm. 50: 483, 1968; Howie et al. Adv. Immunol. 9:215, 1968; *Genetic Control of Autoimmune Disease*, Rose, M., Bigazzi, P.E., and Warner, N.L. eds., Elsevier, Amsterdam, 1979; and *Current Protocols In Immunology*, supra). For example, the NZBxNZW F<sub>1</sub> mouse is an excellent model of human SLE, female mice develop high levels of anti-double- and single-stranded DNA autoantibodies, other anti-nuclear antibodies, and renal disease; death usually occurs at approximately 8 months (see e.g., Theofilopoulos et al., Adv. Immunol. 37:269, 1985).

To evaluate the immunosuppressive effect of rHuAFP or a fragment or analog thereof on SLE, i.e., the compound's ability of rHuAFP to prevent or ameliorate SLE, test compounds are administered to an appropriate animal, e.g., the NZBxNZW F<sub>1</sub> mouse, according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration is initiated prior to the onset of SLE and/or after the clinical appearance of SLE. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test compound on SLE is monitored according to standard methods. For example, analysis of autoantibodies, e.g., anti-DNA antibodies can be monitored. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of kidney tissue is performed and tissue samples examined microscopically for evidence of SLE, e.g., evidence of lupus nephritis.

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Comparative studies between treated and control animals are used to determine the relative efficacy of the test compounds in preventing or ameliorating SLE. A molecule which prevents or ameliorates (decreases or suppresses or  
5 relieves or promotes remission of) the symptoms of SLE is considered useful in the invention.

#### Therapeutic Administration

As demonstrated above, recombinant alpha-fetoprotein, e.g., rHuAFP (or a fragment or analog  
10 thereof) is effective in inhibiting proliferation of autoimmune cells and accordingly is useful for the prevention or amelioration of autoimmune diseases including, but not limited to, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, systemic lupus  
15 erythematosus, and myasthenia gravis. Accordingly, recombinant human alpha-fetoprotein (or a fragment or analog thereof) can be formulated according to known methods to prepare pharmaceutically useful compositions.

Recombinant alpha-fetoprotein, e.g., rHuAFP (or a  
20 fragment or analog thereof), is preferably administered to the patient in an amount which is effective in preventing or ameliorating the symptoms of an autoimmune disease. Generally, a dosage of 0.1 ng/kg to 10 g/kg body is adequate. If desired, administration is performed on  
25 a daily basis. Because there are no known adverse side effects related to recombinant human alpha-fetoprotein, it is believed that relatively high dosages can be safely administered. For example, treatment of human patients will be carried out using a therapeutically effective  
30 amount of rHuAFP (or a fragment or analog thereof) in a physiologically acceptable carrier. Suitable carriers and their formulation are described for example in Remington's Pharmaceutical Sciences by E.W. Martin. The amount of rHuAFP to be administered will vary depending  
35 upon the manner of administration, the age and body

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weight of the patient, and with the type of disease, and size of the patient predisposed to or suffering from the disease. Preferable routes of administration include, for example, subcutaneous, intravenous, intramuscular, or  
5 intradermal injections which provide continuous, sustained levels of the drug in the patient. In other preferred routes of administration, rHuAFP can be given to a patient by injection or implantation of a slow release preparation, for example, in a slowly  
10 dissociating polymeric or crystalline form; this sort of sustained administration can follow an initial delivery of the drug by more conventional routes (for example, those described above). Alternatively, rHuAFP can be administered using an infusion pump (e.g., an external or  
15 implantable infusion pump), thus allowing a precise degree of control over the rate of drug release, or through installation of rHuAFP in the nasal passages in a similar fashion to that used to promote absorption of insulin. As an alternative to nasal transmucosal  
20 absorption, rHuAFP can be delivered by aerosol deposition of the powder or solution into the lungs.

Furthermore, the method(s) of the invention can also employ combination therapy in which rHuAFP is administered either simultaneously or sequentially with a  
25 therapeutic agent such as a general or specific tolerizing agent, e.g., an anti-idiotypic agent (e.g., a monoclonal) or a therapeutic vaccine or an oral agent (e.g., insulin, collagen or myelin basic protein) or a cytokine (e.g., Il-15) or an interferon ( $\alpha$ -interferon) or  
30 an immunosuppressive agent. Preferably, an immunosuppressive agent is administered in an effective dose which is lower than the standard dose when the immunosuppressive agent is used by itself. Preferred immunosuppressive agents are cyclosporine, FK-506,  
35 steroids, azathioprine, or 15-deoxyspergualin.

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Treatment is started generally with the diagnosis or suspicion of an autoimmune disease and is generally repeated on a daily basis. Protection or prevention from the development (or progression or exacerbation) of an autoimmune disease is also achieved by administration of rHuAFP prior to the onset of the disease. If desired, the efficacy of the treatment or protection regimens is assessed with the methods of monitoring or diagnosing patients for autoimmune disease.

10 Recombinant Human AFP in the Treatment and Diagnosis of Cancer

CYTOTOXIC AGENTS

A hybrid cytotoxin of rHuAFP is prepared by conjugating a full-length rHuAFP or a fragment or analog thereof to any number of known toxic entities using conventional techniques. Such toxins are useful for inhibiting the development of a neoplasm (as described infra). Useful cytotoxins are preferably significantly cytotoxic only when present intracellularly and are substantially excluded from any given cell in the absence of a targeting domain. As described below, peptide toxins fulfill both of these criteria and are readily incorporated into hybrid molecules. If desired, a mixed cytotoxin (i.e., a cytotoxin composed of all or part of two or more toxins) can also be used. Several useful toxins are described in more detail below.

Toxin molecules useful in the method of the invention are preferably toxins, such as peptide toxins, which are significantly cytotoxic only when present intracellularly. Of course, under these circumstances the molecule must be able to enter a cell bearing the targeted receptor. This ability depends on the nature of the molecule and the nature of the cell receptor. For example, cell receptors which naturally allow uptake of a ligand are likely to provide a means for a molecule which

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includes a toxin to enter a cell bearing that receptor. As is discussed below, the peptide toxin useful in the methods of the invention is fused to a rHuAFP (or fragment or analog thereof) binding domain by producing a 5 recombinant DNA molecule which encodes a hybrid protein molecule.

Many peptide toxins have a generalized eukaryotic receptor binding domain; in these instances the toxin must be modified to prevent intoxication of non-receptor 10 bearing cells. Any such modifications must be made in a manner which preserves the cytotoxic functions of the molecule (see U.S. Department of Health and Human Services, U.S. Serial No. 401,412). Potentially useful toxins include, but are not limited to: cholera toxin, 15 ricin, O-Shiga-like toxin (SLT-I, SLT-II, SLT II<sub>v</sub>), LT toxin, C3 toxin, Shiga toxin, pertussis toxin, tetanus toxin, Pseudomonas exotoxin, saporin, modeccin, and gelanin.

The cytotoxic portion of some molecules useful in 20 the invention, if desired, can be provided by a mixed toxin molecule. A mixed toxin molecule is a molecule derived from two different polypeptide toxins. Generally, as discussed above, polypeptide toxins have, in addition to the domain responsible for generalized 25 eukaryotic cell binding, an enzymatically active domain and a translocation domain. The binding and translocation domains are required for cell recognition and toxin entry respectively. The enzymatically active domain is the domain responsible for cytotoxic activity 30 once the molecule is inside a cell.

Naturally-occurring proteins which are known to have a translocation domain include diphtheria toxin, Pseudomonas exotoxin A, and possibly other peptide toxins. The translocation domains of diphtheria toxin 35 and Pseudomonas exotoxin A are well characterized (see,



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e.g., Hoch et al., Proc. Natl. Acad. Sci. USA 82:1692, 1985; Colombatti et al., J. Biol. Chem. 261:3030, 1986; and Deleers et al., FEBS Lett. 160:82, 1983), and the existence and location of such a domain in other  
5 molecules may be determined by methods such as those employed by Hwang et al. Cell 48:129, 1987); and Gray et al. Proc. Natl. Acad. Sci. USA 81:2645, 1984).

For example, one useful rHuAFP/mixed toxin hybrid molecule is formed by fusing the enzymatically active A  
10 subunit of E. coli Shiga-like toxin (see, e.g., Calderwood et al., Proc. Natl. Acad. Sci. USA 84:4364, 1987) to the translocation domain (amino acid residues 202 through 460) of diphtheria toxin, and to rHuAFP. The rHuAFP portion of the three-part hybrid causes the  
15 molecule to attach specifically to cells bearing receptors which is recognized by rHuAFP, and the diphtheria toxin translocation portion acts to insert the enzymatically active A subunit of the Shiga-like toxin into the targeted cell. The enzymatically active portion  
20 of Shiga-like toxin, like diphtheria toxin, acts on the protein synthesis machinery of the cell to prevent protein synthesis, thus killing the cell.

Functional components of the hybrid cytotoxins of the invention are linked together via a non-covalent or  
25 covalent bond, or both. Non-covalent interactions can be ionic, hydrophobic, or hydrophilic, such as interactions involved in a leucine-zipper or antibody-protein G interaction (see, e.g., Derrick et al., Nature 359:752, 1992). An example of a covalent linkage is a disulfide  
30 bond.

A hybrid cytotoxin is prepared by chemically conjugating rHuAFP (or fragment or analog) to a any number of known toxic entities, e.g., those described above. Such reactions are carried out by standard  
35 techniques known to those skilled in the art. A typical

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way of conjugating a protein to a protein toxin (including, e.g., bacterial toxins such as diphtheria toxin or Pseudomonas exotoxin A, or plant toxins such as ricin) is by crosslinking through a disulfide bond (see, 5 e.g., Chang et al., J. Biol. Chem. 252:1515, 1977) or a heterobifunctional molecule (see, e.g., Cawley et al. Cell 22:563, 1980). See also Stevens et al., U.S. Pat. No. 4,894,227.

Alternatively, the hybrid cytotoxin is prepared by 10 expression of a hybrid DNA engineered to encode both the rHuAFP (or a fragment or analog thereof) and the toxin (or a toxic portion thereof), using technology available to those of ordinary skill in the art of making such hybrids (see, e.g., Murphy, U.S. Pat. No. 4,675,382, and 15 Chadhary et al., Proc. Natl. Acad. Sci. USA 84:4538, 1987). For example, a recombinant fusion protein of rHuAFP and a cytotoxic agent is made according to methods known in the art (see, e.g., Murphy supra and Huston et al., Meth. Enzymol. 203:46, 1991). If the hybrid 20 cytotoxin is produced by expression of a fused gene, a peptide bond serves as the link between the cytotoxic agent and the targeting ligand. Another method useful for conjugating a protein or polypeptide to a protein toxin employs the polymer, monomethoxy-polyethylene 25 glycol (mPEG), as described in Maiti et al., Int. J. Cancer Suppl. 3:17, 1988.

If desired, following its synthesis, the hybrid cytotoxin is affinity purified according to standard methods using antibodies against the targeting portion of 30 the molecule, e.g., antibodies against human alpha-fetoprotein. Similarly, antibodies directed against the cytotoxic agent are also useful for purifying the hybrid cytotoxin molecule by standard immunological techniques. The resulting hybrid cytotoxin is then formulated for use 35 as an agent against unwanted cells, e.g. cancer cells,

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following procedures standard in the field of pharmacology.

Molecules of the invention can be screened for the ability to decrease viability of cells bearing the targeted receptor by means of assays known in the art, e.g., those methods described herein.

Because hybrid cytotoxins of the invention are potent cytotoxic agents for cells bearing the a receptor which is recognized by rHuAFP, rHuAFP is useful in the treatment of diseases involving unwanted alpha-fetoprotein receptor-positive cells, e.g., cancer cells.

#### DIAGNOSTIC AGENTS

Recombinant rHuAFP or a fragment or analog thereof can be attached to a detectable label to produce an agent useful for detecting and localizing a neoplasm in vivo, in situ, or in vitro. Methods for attaching such labels to proteins are known in the art. For example, a detectable label is attached by chemical conjugation, but where the label is a polypeptide, it could alternatively be attached by genetic engineering techniques.

Detectable labels are generally selected from a variety of such labels known in the art, but are normally radioisotopes, fluorphores, enzymes (e.g., horseradish peroxidase), or other moieties or compounds which emit a detectable signal (e.g., radioactivity, fluorescence, color) or emit a detectable signal after exposure of the label to its substrate. Various detectable label/substrate pairs (e.g., horseradish peroxidase/diaminobenzidine, avidin/streptavidin, luciferase/luciferin,  $\beta$ -galactosidase/X-gal(5-Bromo-4-Chloro-3-Indoyl-D-Galactopyranoside), and methods for labelling proteins for such detection purposes are known in the art. The usefulness of such an agent can be assayed, for example, by implanting a tumor cell line, e.g., MCF-7, into a host, e.g., a mouse, and determining

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whether the agent of the invention detectably labels the tumor produced by such implanted cells, e.g., by radioimaging using scintigraphy. Such an agent can also be used to assay for the presence of any unwanted cell  
5 bearing an alpha-fetoprotein receptor, e.g., by using Western blot analysis or histochemical staining of a tissue sample, according to known methods.

Recombinant HuAFP as an Anti-Cancer Agent

Anti-cancer agents of the invention (e.g., rHuAFP  
10 or a fragment or analog thereof; or a hybrid cytotoxin of rHuAFP) are useful for inhibiting a neoplasm, e.g., breast or prostate carcinomas. Those skilled in the art will understand that any number of methods, both in vitro and in vivo, are used to determine the efficacy of anti-  
15 cancer agents useful in the methods of the invention. For example, the reduction of tumor growth can be monitored in a mouse or rat growing a prostate cancer (e.g., tumor xenografts of LNCaP androgen receptor-positive human prostate cancer cell line) following the  
20 administration of the test compound. In a working example, a human tumor cell lines (e.g., cell lines such as MCF-7(ATCC HTB 22), T-47D (ATCC HTB 133), MDA-MB-231 (ATCC HTB 26), BT-20 (ATCC HTB 19), NIH:OV-CAR-3 (ATCC HTB 161), LnCaP.FGC (ATCC CRL 1740), and Du-145 (ATCC HTB  
25 81) growing in culture is released from monolayer by trypsinization, diluted into single-cell suspension and then solidified by centrifugation into a pellet which IS subsequently exposed to 15  $\mu$ l fibrinogen (50 mg/ml) and 10  $\mu$ l thrombin (50 units/ml) for 30 minutes at 37°C.  
30 Fibrin clots containing tumor are then cut into pieces approximately 1.5 mm in diameter. Each piece of tumor is subsequently implanted under the kidney capsule of a mouse according to standard methods. If desired, mice can be immunosuppressed by daily subcutaneous (s.c.)  
35 injection of 60 mg/kg cyclosporine A (Sandimmune IV)

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- beginning immediately prior to tumor implantation according to conventional methods. If necessary, estrogen and androgen supplementation of mice is achieved by standard methods, e.g., implantation of silastic tubing containing estradiol or by injection of testosterone propionate. Typically, hormone supplementation is commenced on the day of tumor implantation. Generally, administration of the test molecule is initiated prior to tumor implantation and/or after tumor implantation.
- 10 Control animals receive a placebo, e.g., human serum albumin or diluent, similarly administered as for rHuAFP or related molecules. The effect of the test molecule on tumor growth is monitored according to any standard method. For example, tumor growth is monitored by weekly
- 15 measurement of tumor size by laparotomy using a dissecting microscope equipped with an ocular micrometer. A molecule shown experimentally to halt or reduce or inhibit the growth of such implanted tumors is considered useful in the invention.
- 20 Toxicity of test compounds towards cells bearing receptors that are recognized by rHuAFP can be tested in vitro according to any standard protocol. For example, a cultured cancer cell line, e.g., MCF-7 estrogen-receptor-positive human breast cancer cell line, is
- 25 maintained in plastic tissue culture flasks (Costar) in DMEM with penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), 5% fetal calf serum, insulin (10 ng/ml), L-glutamine (2 mM) and non-essential amino acids (1%). Cells are seeded in 96-well V-bottomed plates (Linbro-
- 30 Flow Laboratories, McLean, VA) at a concentration of  $1 \times 10^5$  per well in complete medium. Putative toxins are added to varying concentrations ( $10^{-12}$ M to  $10^{-6}$ M), and the cultures are incubated for 18 hrs. at 37°C in a 5% CO<sub>2</sub> atmosphere. Following incubation, the plates are
- 35 centrifuged for 5 min. at 170 x g, and the medium removed

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and replaced with 100  $\mu$ l leucine-free medium (MEM, Gibco) containing 8  $\mu$ Ci/ml ( $^3$ H-leucine; New England Nuclear, Boston, MA). After an additional 90 min. at 37°C, the plates are centrifuged for 5 min. at 170 x g, the medium  
5 is removed, and the cells are collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters are washed, dried, and counted according to standard methods. Cells cultured with medium alone serve as the control. A test compound which reduces or halts  
10 or inhibits cell growth compared to untreated control cells, is detected as an indication of toxicity and is considered useful in the invention.

Evaluation of whether a test compound confers protection against the development of a neoplasm (e.g.,  
15 breast or prostate cancers) generally involves using an animal known to develop a neoplasm (e.g., the transgenic mouse described in U.S. Pat. No. 4,736,866). An appropriate animal is treated with the test compound according to standard methods, and a reduced incidence of  
20 neoplasm development, compared to untreated control animals, is detected as an indication of protection.

As is discussed below, I have discovered that unglycosylated rHuAFP produced in a prokaryotic expression system is effective in treating cancer. For  
25 example, rHuAFP has been found to be a potent inhibitor of breast carcinoma growth in vitro.

The experimental examples described below demonstrate the efficacy of rHuAFP as an anti-cancer agent. These examples are provided to illustrate, not  
30 limit, the invention.

#### EXPERIMENTAL

##### MATERIALS AND METHODS

##### Culture Media and Tumor Cells

Dulbecco's modified Eagle's medium (DMEM), RPMI  
35 1640, fetal calf serum, glutamine, non-essential amino

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acids and penicillin-streptomycin mixture were obtained from GIBCO (BRL). Donor calf serum was obtained from Hyclone, Logan, UT, and porcine insulin was obtained from Squibb, Inc., Princeton, NJ.

5           The MCF-7 estrogen-receptor-positive human breast cancer cell line was obtained from Dr. Alberto C. Baldi, Institute of Experimental Biology and Medicine, Buenos Aires, Argentina. Stock cultures were maintained in plastic tissue culture flasks (Costar) in DMEM with  
10 penicillin (100 U/ml), streptomycin (100 µg/ml), 5% fetal calf serum, insulin (10 ng/ml), L-glutamine (2 mM) and non-essential amino acids (1%).

Estrogen-Stimulated Post-confluent Growth of MCF-7 Cells in Culture.

15           This assay is based on the finding that MCF-7 cells in estrogen-containing medium grow past confluence and accumulate into foci; but, in the absence of estrogen, cell proliferation stops after the cultures establish cell-cell contact, and no foci are formed (see,  
20 e.g., Gierthy et al., Breast Cancer Res. Treat. 12:227, 1988).  $1 \times 10^7$  MCF-7 breast cancer cells were seeded in 16-mm wells contained in 24-well tissue culture plates. Culture medium was phenol red-free DMEM supplemented with 5% donor calf serum (prescreened for absence of  
25 detectable estrogens), L-glutamine (2 mM), non-essential amino acids (1X, GIBCO), insulin (10 ng/ml), penicillin-streptomycin (1X, GIBCO) and estradiol diluted to a final concentration of  $1.8 \times 10^{-9}$  M. Cultures were refed at 24 hr and every 4 days thereafter with 2 ml of culture  
30 medium containing rHuAFP and human serum albumin to yield a final protein concentration of 100 µg/ml per well. Cells reached confluence within 5 days, and a substantial number of foci were apparent within 10 days in wells containing estrogen alone. Cells were fixed with  
35 buffered formalin and stained with 1% Rhodamine B. The

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stained foci were quantitated using an Artek 870 Macro-Micro Automated Colony Counter. Data are presented as mean number of foci per treatment group.

### RESULTS

#### 5           Activity of rHuAFP Against MCF-7 Breast Cancer Cells

The results shown in Fig. 9 demonstrate that rHuAFP inhibits estrogen-stimulated postconfluent growth of MCF-7 breast cancer cells in vitro. Control  
10 experiments using human albumin or no protein had no effect on MCF-7 foci formation. These data indicate that rHuAFP has a direct inhibitory effect on the growth of carcinoma cell cultures.

#### Therapeutic Administration

15           As demonstrated above, rHuAFP is effective in inhibiting a neoplasm, e.g., a breast cell carcinoma. Accordingly, compounds of the invention can be formulated according to known methods to prepare pharmaceutically useful compositions. Treatment of human patients will be  
20 carried out using a therapeutically effective amount of an anti-cancer agent of rHuAFP in a physiologically acceptable carrier. Suitable carriers and their formulation are described for example in Remington's Pharmaceutical Sciences by E.W. Martin. The amount of  
25 the anti-cancer agent to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the type of disease, extensiveness of the disease, and size of the patient suffering from the disease. Generally amounts will be in  
30 the range of those used for other agents used in the treatment of cancer, although in certain instances lower amounts will be needed because of the increased specificity of the compound. For example, rHuAFP is administered systemically, as described below, at a



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dosage that inhibits malignant cell proliferation, typically in the range of 0.1 ng - 10 g/kg body weight.

Furthermore, the method of the invention can also employ combination therapy in which rHuAFP is

5 administered either simultaneously or sequentially with a chemotherapeutic agent. Typically, a chemotherapeutic agent is administered according to standard methods or, alternatively, in a dose which is lower than the standard dose when the chemotherapeutic agent is used by

10 itself. Examples of chemotherapeutic agents include, without limitation, mechlorethamine, cyclophosphamide, ifosfamide, L-sarcolysin, chlorambucil, hexamethylmelamine, thiotepa, busulfan, carmustine, lomustine, semustine, streptozocin, dacarbazine,

15 methotrexate, fluorouracil, cytarabine, mercaptopurine, thioguanine, pentostatin, vinblastine, vincristine, etoposide, teniposide, actinomycin D, daunomycin, doxorubicin, bleomycin, plicamycin, mitomycin, cisplatin, mitoxantrone, hydroxyurea, procarbozine, mitotane,

20 aminoglutethimide, prednisone, hydroxyprogesterone, diethylstilbestrol, tamoxifen, flutamide, or leuprolide.

Treatment is started generally with the diagnosis or suspicion of a neoplasm and is generally repeated on a daily basis. Protection from the development of neoplasm

25 is also achieved by administration of rHuAFP on a daily basis. If desired, the efficacy of the treatment or protection regimens is assessed with the methods of monitoring or diagnosing patients for cancer.

Furthermore, the compounds of the invention can

30 also be used to treat mammals to destroy any unwanted cells bearing alpha-fetoprotein receptors associated with a pathological condition. The method(s) of the invention can also be used to treat non-human mammals, for example, domestic pets, or livestock. As described below, the

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anti-cancer agents of the invention can be administered systemically or locally.

#### Systemic Administration

For use as an anti-cancer agent, the compounds of the invention can be administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such as physiological saline. Preferable routes of administration include, for example, subcutaneous, intravenous, interperitoneally, intramuscular, or intradermal injections which provide continuous, sustained levels of the drug in the patient. In other preferred routes of administration, the compounds of the invention can be given to a patient by injection of a slow release preparation, for example, in a slowly dissociating polymeric or crystalline form; this sort of sustained administration can follow an initial delivery of the drug by more conventional routes (for example, those described above). Alternatively, the compounds can be administered using an infusion pump, thus allowing a precise degree of control over the rate of drug release, or through installation of the compounds in the nasal passages in a similar fashion to that used to promote absorption of insulin. As an alternative to nasal transmucosal absorption, the compounds can be delivered by aerosol deposition of the powder or solution into the lungs.

#### Local Administration

The anti-cancer agents of the invention also can be administered locally to treat cancer. Since the desired action of the agent is generally upon a circumscribed mass of tissue, for example a tumor, delivery of the drug by means which result in high local concentrations in the vicinity of the tumor is especially desirable.

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Recombinant HuAFP as a Diagnostic Agent

Recombinant HuAFP (or fragment or analog thereof) linked to a detectable label finds diagnostic use in the detection or monitoring or assaying for the presence of a  
5 neoplasm (e.g., breast or prostate cancers).

For example, in vivo studies can be conducted on human patients to determine the presence of a neoplasm using a detectably labelled rHuAFP (e.g., Tc-99m-labelled rHuAFP). In general, the detectably labelled rHuAFP is  
10 administered intravenously and imaging can be performed using scanners by methods known to those skilled in the art, e.g., by radioimaging using scintigraphy.

In another working example, a neoplasm or any cell bearing a receptor which is recognized by rHuAFP may be  
15 detected in a tissue sample, e.g., a biopsy, a bodily fluid, by using rHuAFP (or fragment or analog thereof) linked to a detectable label. After determining that a patient should be tested for the presence of such cells, a tissue sample, a biopsy, or a sample of bodily fluid,  
20 preferably lymph, blood, serum, or urine, is collected from the patient. Accordingly, the subcellular location or presence of a receptor which is recognized by rHuAFP is determined either in situ or in vitro using fractionated cells by any standard biochemical or  
25 histochemical procedure (see e.g., Ausubel et al., supra; Bancroft and Stevens, Theory and Practice of Histological Techniques, Churchill Livingstone, 1982). Appropriate control samples for the assay include a tissue sample or a bodily fluid collected from individuals who do not have  
30 cells bearing alpha-fetoprotein (negative control), or samples which contain a known, predetermined amount of alpha-fetoprotein receptor (positive control).

The diagnostic assay may be performed in solution or may use a solid (insoluble) support (e.g. polystyrene,  
35 nitrocellulose, or beads) or in a tissue sample prepared

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for histological examination, using any standard methods. For example, to determine whether the patient from whom the test sample was collected has cells bearing receptors which is recognized by rHuAFP, the level of binding of the detectably labelled rHuAFP in the test sample is compared to the level of binding in the negative and/or positive control samples. A level of binding in the test sample greater than the level of binding in the negative control sample, or at least equal to the level of binding in the positive control sample, indicates that the patient has cells bearing alpha-fetoprotein receptors.

Materials for performing the diagnostic assays according to the methods of the invention may be provided as a kit having instructions for use. In general, the kit is composed in part of a rHuAFP (or fragment or analog thereof). This kit may further include a second reagent, e.g., a detectable label, which is used to label rHuAFP (or a fragment or analog thereof). The kits exemplified above are useful in, for example, detecting the presence of a tumor in a sample of human tissue in vitro, or for in vivo examination purposes.

The experimental examples described below demonstrate the efficacy of rHuAFP diagnosing a neoplasm. These examples are provided to illustrate, not limit, the invention.

## EXPERIMENTAL

### MATERIALS AND METHODS

#### Animals

MCF-7 human breast cancer cells implanted in the lateral thorax region of CB-17 SCID mice were grown to a size of 1 cm diameter (approx. 5 gm) under estrogen stimulation according to methods known in the art.

#### Technetium Labelling

<sup>99m</sup>Tc-recombinant labelled alpha-fetoprotein was prepared from an AFP aliquot mixed with 0.5 ml 0.9%

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sodium chloride injection solution (Baxter Healthcare Corporation, Deerfield, IL). The solution is added to an UltraTag RBC Reaction Vial (Mallinckrodt Medical Inc., St. Louis, MO 63134 Lot No. 0683040), containing stannous chloride dihydrate, sodium citrate dihydrate, and dextrose anhydrous, in a lyophilized form stored under argon. The contents of the vial are mixed by gentle swirling, and incubated at room temperature for 5 minutes. At the completion of the incubation, 0.8-1.2 Gbq Technetium 99mTc Sodium Pertechnetate Injection is added (99mTc Generator Mallinckrodt Medical, Inc. St. Louis, MO) in a volume of 1-2 ml. The contents of the vial are mixed by gentle swirling and incubated for 15 minutes. Dose aliquots were assayed at 0, 3, and 6 hours after preparation. Thin-layer chromatography performed on preparations using ITLC-SG (Gelman Instrument Co., Ann Arbor, MI) with 0.9% NaCl showed 95-99% of the 99Tc was bound to the recombinant alpha-fetoprotein.

#### Imaging

Experimental animals are sedated with Medafane. A 24 gauge, 3/4 inch catheter (Surflo IV catheter, Terumo Medical) is then secured in a lateral tail vein. The animal is then further anesthetized with a slow infusion of 20-25 mg/kg body weight of pentobarbital intravenously. Anesthesia is maintained for restraint as required with injections of 5 mg of additional pentobarbital.

Isotope biodistribution data is collected using a Elscint Dymax 409 gamma camera. This data is subsequently analyzed by a computer (Siemens Gammasonics Microdelta). Animals are imaged in triples, being placed in the dorsal recumbent position on a thin polyethylene panel. To eliminate motion during imaging, the animals are restrained as necessary, on these panels by strips of tape over their extremities so as not to restrict

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respiration. Dynamic images obtained over 60 minutes are used to determine the biodistribution of the labeled protein. Typically, twelve sequential, five minute images are obtained with low energy general purpose collimation, and 1.5 hardware zoom into the computer matrices having 128 by 128 picture elements. Study animals are typically injected with 37MBq of Tc-99m labelled protein.

### RESULTS

#### 10           Tracer Biodistribution and Kinetics

Following administration of 37MBq (approx. 4-6  $\mu$ g Tc-99m recombinant human alpha-fetoprotein) in the tail vein, tracer biodistribution kinetics were measured during the initial hour after injection and at 24 hours.

15 Tissue uptake kinetics were measured in % injected activity/per 100 ROI (Region of Interest) pixels (%IA). During the first hour there is rapid renal clearance, mild localization in the liver and little evident activity in other tissues. At 1 hour, tumor uptake was

20 (mean  $\pm$  SEM:  $1.9 \pm 0.3\%$ IA) and the tumor to heart (T/H) region ratio was  $0.84 \pm 0.23$ . By 24 hours, tumor uptake was ( $0.8 \pm 0.1\%$ IA) and T/A and tumor to background (T/B upper chest) region ratios were  $1.43 \pm 0.41$  and  $2.66 \pm 0.54$ , respectively. Studies comparing 99mTc-labelled

25 rHuAFP to 99mTc-labelled human serum albumin (used as a non-specific protein control) repeated in the same animals showed that T/B image ROI activity ration was 2.7 and 5.8 for 99mTc-labelled rHuAFP at 1 and 24 hr, respectively and at 24 hours was 40% greater for 99mTc-

30 labelled rHuAFP compared to Tc-99m human serum albumin. These results show that rHuAFP can be labelled with Tc-99m and that this labelled agent has low non-specific tissue uptake and rapid renal clearance from the blood. Localization in human breast cancer xenografts is

35 initially rapid, increases with time, and is due to

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specific tumor uptake. These results demonstrate that rHuAFP labelled with Tc-99m is useful as a diagnostic agent for breast carcinoma.

#### Diagnostic Administration

5 As discussed above, rHuAFP (or fragment or analog thereof) linked to a detectable label finds diagnostic use in the detection or monitoring or assaying of a neoplasm (e.g., a breast cell carcinoma). Accordingly, patients who present with the classical symptoms of  
10 cancer, e.g., breast cancer or prostate cancer, or have a medical history which indicates susceptibility to such cancer may be tested with the methods of the invention. Other appropriate patients for such testing include those who have a family history of breast or prostate  
15 carcinomas. Patients who are receiving drugs or have been exposed to toxins implicated in the induction of a cancer should also be tested.

The diagnostic methods employing detectably labelled rHuAFP (or a fragment or analog thereof) of the  
20 invention may be used to detect the presence of a cancer prior to, or after the onset of, clinical symptoms associated with the cancer.

The method of the invention facilitates diagnosis of a neoplasm prior to or coincident with the onset of  
25 clinical symptoms (e.g., a palpable tumorous mass). For example, the method of the subject invention may provide a diagnosis of breast cancer prior to onset of clinical symptoms. Furthermore, the method of the invention allows the clinician to provide an accurate diagnosis of  
30 a neoplasm such as breast or prostate cancer.

Diagnostic imaging methods of the invention will be carried out using a diagnostically effective amount of a diagnostic agent of rHuAFP in a physiologically acceptable carrier. Suitable carriers and their  
35 formulation are described for example in Remington's

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Pharmaceutical Sciences by E.W. Martin. The amount of the diagnostic agent to be administered varies depending upon the manner of administration, the age and body weight of the patient, and with the type of disease, 5 extensiveness of the disease, and size of the patient suffering from the disease. Generally, however, amounts will be in the range of those used for other agents used in the diagnosis of cancer, although in certain instances lower amounts will be needed because of the increased 10 specificity of the compound. For example, a detectably labelled rHuAFP is administered intravenously to a patient, as is described above, at a dosage that allows imaging of a neoplasm, e.g., by radioimaging using scintigraphy. Typically, a dosage is in the range of 0.1 15 ng - 10 g/kg body weight.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and 20 individually indicated to be incorporated by reference.

Cell Culture Media of the Invention

The invention further provides a media containing rHuAFP (or a fragment or analog thereof) for cell culture. While media of the invention generally does not 25 require the use of serum (e.g., fetal bovine serum, calf serum, horse serum, normal mouse serum, human serum, porcine serum, rabbit serum etc.), since such rHuAFP is intended to replace or supplement the use of serum, those skilled in the art will understand and recognize that 30 serum can be added if desired. Media formulations are generally prepared according to methods known in the art. Accordingly, any standard medium, e.g., RMPI-1630 Medium, CMRL Medium, Dulbecco's Modified Eagle Medium (D-MEM), Fischer's Medium, Iscove's Modified Dulbecco's Medium, 35 McCoy's Medium, Minimum Essential Medium, NCTC Medium,



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and the like can be formulated with rHuAFP (or a fragment or analog thereof) at the desired effective concentration. If desired, media supplements, e.g., salt solutions (e.g., Hank's Balanced Salt Solution or Earle's  
5 Balanced Salt Solution), antibiotics, nucleic acids, amino acids, carbohydrates, and vitamins are added according to known methods. If desired, growth factors, colony-stimulating factors, cytokines and the like can also be added to media according to standard methods.

10 For example, media of the invention can contain any of the following substances, alone or in combination, with rHuAFP (or a fragment or analog thereof): erythropoietin, granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF),  
15 macrophage colony-stimulating factor (M-CSF), an interleukin (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, etc.), insulin-growth factor (IGF), transferrin, albumin, and stem-cell growth factor (SCF). Media of the invention are useful for culturing a variety of eukaryotic cells,  
20 e.g., mammalian cells, yeast cells, amphibian cells, and insect cells. Media can also be used for culturing any tissue or organ. Such media can also be used in a variety of culture conditions and for a variety of biological applications. Examples of such culture  
25 conditions include, without limitation, bioreactors (e.g., continuous or hollow fiber bioreactors), cell-suspension cultures, semisolid cultures, liquid cultures, and long-term cell suspension cultures. Media of the invention are also useful for industrial applications,  
30 e.g., culturing hybridoma cells, genetically-engineered mammalian cells, tissues or organs.

Recombinant Human Alpha-Fetoprotein As A Cell-  
Proliferative Agent

Cell growth-promoting attributes of rHuAFP (or a  
35 fragment or analog thereof) is evaluated by any standard

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assay for analysis of cell proliferation in vitro and in vivo. As discussed infra, the art provides animal systems for in vivo testing of cell growth promoting or boosting characteristics of rHuAFP (or a fragment or analog thereof). Furthermore, a wide variety of in vitro systems are also available for testing growth-promoting or growth-boosting aspects of rHuAFP (or a fragment or analog thereof).

Any cell that proliferates in response to rHuAFP (or a fragment or analog thereof) can be identified according to standard methods known in the art. For example, proliferation of a cell (e.g., a bone marrow cell) can be monitored by culturing in a liquid media containing the test compound, either alone or in combination with other growth factors, added artificially to a serum-free or serum-based medium. Alternatively, such bone marrow cells can be cultured in a semisolid matrix of dilute agar or methylcellulose, and the test compound, alone or in combination with other growth factors, can be added artificially to a serum-free or serum-reduced medium. In the semisolid matrix the progeny of an isolated precursor cell, proliferating in response to rHuAFP or a fragment or analog thereof, remain together as a distinguishable colony. For example, a bone marrow cell may be seen to give rise to a clone of a plurality of bone marrow cells, e.g., NK cells. Such culture systems provide a facile way for assaying whether a cell responds to rHuAFP (a fragment or analog thereof) either alone or in combination with other growth factors.

If desired, identification and separation of expanded subpopulations of cells is performed according to standard methods. For example, cells may be analyzed by fluorescence-activated cell sorting (FACS). This procedure generally involves labelling cells with

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antibodies coupled to a fluorescent dye and separating the labeled cells from the unlabelled cells in a FACS, e.g., FACScan (Becton Dickson). Thus virtually any cell can be identified and separated, e.g., by analyzing the presence of cell surface antigens (see e.g., Shah et al., J. Immunol. 140:1861, 1988). When a population of cells is obtained, it is then analyzed biochemically or, alternatively, provides a starting population for additional cell culture, allowing the action of the cells to be evaluated under defined conditions in culture.

In one working example, the effect of rHuAFP (or a fragment or analog thereof) on the growth of human bone marrow cells is examined as follows. In general, human bone marrow samples are obtained according to standard procedures after informed consent. For example, bone marrow is obtained from the iliac crest of a healthy donor and the marrow cells are diluted in phosphate-buffered saline at room temperature. Cells are then washed and cultured in an appropriate growth medium. For example, cultures can be set up by inoculating bone marrow cells in 20-30 ml of McCoy's medium containing 50 U/ml penicillin, 50 U/ml streptomycin and 2 mM L-glutamine. Cultures are incubated in the presence or absence of the test compound alone, or in combination with other growth factors, e.g., transferrin or GM-CSF. The cultures are subsequently incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, 5% O<sub>2</sub>, and 90% N<sub>2</sub> for the desired time period. Cell proliferation assays are performed according to standard methods. For example, replicate samples cultured in the presence and absence of the test compound are analyzed by pulsing the cells with 1-2 µCi of <sup>3</sup>HTdR. After an incubation period, cultures are harvested onto glass-fiber filters and the incorporated <sup>3</sup>H measured by liquid scintillation. Comparative studies between treated and control cells,

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e.g., cell cultured in the presence of rHuAFP versus cells cultured in the absence of rHuAFP, are used to determine the relative efficacy of the test molecule in stimulating cell proliferation. A molecule which  
5 stimulates cell proliferation is considered useful in the invention.

To evaluate the proliferative effects of rHuAFP (or a fragment or analog thereof) e.g., the effect of the test compound on hematopoiesis in vivo, the test molecule  
10 is administered to sublethally irradiated mice (or mice treated with an immunosuppressive agent such as cyclosporine or FK-506, or a chemotherapeutic agent such as 5-fluorouracil or cyclophosphamide or any other method known in the art to deplete bone marrow) and normal mice  
15 according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration of the test compound to treated mice is initiated prior to and/or after treating the animal, e.g., with sublethal radiation or  
20 immunotherapy or chemotherapy. Control animals receive a placebo, e.g., human serum albumin or diluent, similarly administered as for rHuAFP or related molecules. The effect of the test molecule on hematopoiesis is monitored by standard techniques. For example, white blood cell  
25 count in peripheral blood and spleen in both treated and control animals are analyzed. Qualitative and quantitative analyses of bone marrow, e.g., lymphocytic lineage or myeloid lineage or any other cell type, can also be determined and analyzed according to conventional  
30 methods. Comparative data between treated and control animals are used to determine the relative efficacy of the test molecule in promoting cell proliferation, e.g., stimulates bone marrow cell production, mature B lymphocyte, thymocyte, or peripheral T lymphocyte cell

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production. A test molecule which stimulates cell proliferation is considered useful in the invention.

The following example demonstrates that unglycosylated rHuAFP stimulates the growth of bone marrow cells in vitro. This example is provided to illustrate, not limit, the invention.

#### EXPERIMENTAL

##### MATERIALS AND METHODS

###### Animals

10 Adult male and female CBA/J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). All mice were bred and maintained in our animal facility. Animals used in this study were 12 to 20 weeks old.

###### Cultures

15 Bone marrow cells were collected by flushing the tibias and femurs of CBA/J mice with modified Dulbecco's phosphate-buffered saline (PBS) using a sterile syringe and 25-gauge needle. Homogenous single-cell suspensions were obtained by the repeated passage of cell mixtures  
20 through a Pasteur pipet. All cells were washed twice by centrifugation at 250g for 10 min in PBS and then assessed for viability by trypan blue dye exclusion. A cell viability of 95% or better was recorded in all experiments. Cells were then adjusted to the desired  
25 concentration prior to use. Bone marrow cells (250,000) were cultured in 96-well round-bottom microtiter plates (Flow Laboratories, Mississauga, Ontario, Canada). The culture medium was serum-free RPMI plus 4 mM L-glutamine, 20 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin  
30 (GIBCO Laboratories, Burlington, Ontario, Canada), 5 µg/ml transferrin, and  $5 \times 10^{-5}$  2-mercaptoethanol (Eastman Chemicals Co., Rochester N.Y.). Cells were cultured in the presence or absence of rHuAFP at a concentration of 400 µg/ml, respectively. Total volume of all cultures  
35 was 0.2 ml. Cultures were maintained at 37°C in 95%

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humidified air and 5% CO<sub>2</sub>. Six hours prior to harvesting, the cultures were pulsed with 1  $\mu$ Ci tritiated thymidine (NEN, sp act 77.1 Ci/mmol). Cells were then harvested on glass fiber mats (Flow Labs) with a multiple sample harvester (Skatron, Flow Labs). Water-insoluble tritiated thymidine incorporation was measured with an LKB 1215 Rackbeta II using standard liquid scintillation techniques.

### RESULTS

#### 10        Effects of rHuAFP on Bone Marrow Proliferation in Serum-Free Media

The effects of purified rHuAFP on cultured murine bone marrow was evaluated in serum-free medium. In this experiment, 2.5 X 10<sup>5</sup> viable cells from bone marrow of CBA/J mice were cultured for 72 hours in serum-free RPMI media in the presence or absence of rHuAFP at a final concentration of 400  $\mu$ g/ml and transferrin at a final concentration of 5  $\mu$ g/ml. Data shown in Fig. 10 indicate that bone marrow cells undergo a strong proliferative response in the presence of unglycosylated rHuAFP; with a stimulation index (SI) of 35. No such proliferation was observed when bone marrow cells were cultured in the absence of rHuAFP.

#### Therapy

25        As demonstrated above, rHuAFP is effective in promoting the proliferation of cells and accordingly is useful for therapy involving the promotion of cell proliferation, e.g., proliferation of bone marrow cells, and in treatment for the prevention of side effects of immunosuppressive therapy, radiotherapy or chemotherapy, or other therapies known to depress the immune system and suppress bone marrow production, causing myelotoxicity. Accordingly, rHuAFP (or a fragment or analog thereof) is employed to treat deficiencies in hematopoietic progenitor or stem cells, or related disorders.

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Recombinant HuAFP (or a fragment or analog thereof) may also be employed in methods for treating cancer and other pathological states resulting in myelotoxicity, exposure to radiation or drugs, and including for example,  
5 leukopenia, bacterial and viral infections, anemia, B cell or T cell deficiencies, including immune cell or hematopoietic cell deficiency following autologous or non-autologous bone marrow transplantation. Recombinant HuAFP (or a fragment or analog thereof) may also be  
10 employed to stimulate development of megakaryocytes and natural killer cells in vitro or in vivo.

The media, compositions, and methods of the invention are also useful for treating cancers that are treated by bone marrow transplants (BMT) that involve  
15 removing bone marrow cells from the patient, maintaining these cells in an ex vivo culture while the patient is treated with radiation or chemotherapy, and then transplanting these cells back into the patient after the treatment has been completed to restore the patient's  
20 bone marrow. Accordingly, rHuAFP may be employed for BMT as a means for reconstituting bone marrow in ex vivo cell culture medium and for promoting bone marrow cell proliferation in vivo. Recombinant HuAFP (a fragment or analog thereof) is also useful for other cell therapies,  
25 e.g. cell expansion and/or gene therapy protocols, therapies requiring ex vivo cell culture. Recombinant HuAFP (a fragment or analog) is also useful in the prevention of autologous or allogenic bone marrow transplant rejection.

### 30        Therapeutic Administration

Recombinant HuAFP (or a fragment or analog thereof) can be formulated according to known methods to prepare pharmaceutically useful compositions. Recombinant human alpha-fetoprotein, e.g., rHuAFP (or a  
35 fragment or analog thereof), is preferably administered

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to the patient in an amount which is effective in preventing or ameliorating the symptoms of myelotoxicity. Generally, a dosage of 0.1 ng/kg to 10 g/kg body weight is adequate. For example, treatment of human patients

5 will be carried out using a therapeutically effective amount of rHuAFP (or a fragment or analog thereof) in a physiologically acceptable carrier. Suitable carriers and their formulation are described for example in Remington's Pharmaceutical Sciences by E.W. Martin. The

10 amount of rHuAFP to be administered will vary depending upon the manner of administration, the age and body weight of the patient, and with the type of disease, and size of the patient predisposed to or suffering from the disease. Preferable routes of administration include,

15 for example, oral, subcutaneous, intravenous, intraperitoneally, intramuscular, transdermal or intradermal injections which provide continuous, sustained levels of the drug in the patient. In other preferred routes of administration, rHuAFP can be given

20 to a patient by injection or implantation of a slow release preparation, for example, in a slowly dissociating polymeric or crystalline form; this sort of sustained administration can follow an initial delivery of the drug by more conventional routes (for example,

25 those described above). Alternatively, rHuAFP can be administered using an external or implantable infusion pump, thus allowing a precise degree of control over the rate of drug release, or through installation of rHuAFP in the nasal passages in a similar fashion to that used

30 to promote absorption of insulin. As an alternative to nasal transmucosal absorption, rHuAFP can be delivered by aerosol deposition of the powder or solution into the lungs.

The therapeutic method(s) and compositions of the

35 present invention may also include co-administration with



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other human growth factors. Exemplary cytokines or hematopoietins for such use include, without limitation, factors such as an interleukin (e.g., IL-1), GM-CSF, G-CSF, M-CSF, tumor necrosis factor (TNF), transferrin, and erythropoietin. Growth factors like B cell growth factor, B cell differentiation factor, or eosinophil differentiation factors may also prove useful in co-administration with rHuAFP (or a fragment or analog thereof). The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

Treatment is started generally with the diagnosis or suspicion of myelotoxicity and is generally repeated on a regular or daily basis to ameliorate or prevent the progression or exacerbation of the condition. Protection or prevention from the development of a myelotoxicemic condition is also achieved by administration of rHuAFP prior to the onset of the disease. If desired, the efficacy of the treatment or protection regimens is assessed with the methods of monitoring or diagnosing patients for myelotoxicity.

The method(s) of the invention can also be used to treat non-human mammals, for example, domestic pets, or livestock.

#### Other Embodiments

In other embodiments, the invention includes the use of rHuAFP (or fragment or analog thereof) for the prevention or treatment of acquired immunodeficiency syndrome (AIDS). To evaluate the immunosuppressive effect of rHuAFP or a fragment or analog thereof on AIDS, i.e., the compound's ability to prevent or ameliorate an autoimmune component of AIDS, test compounds are administered to an appropriate animal (e.g., a human patient), according to standard methods, e.g.,

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intravenously or intraperitoneally, at an appropriate dosage on a daily basis as is discussed above.

Generally, administration is initiated prior to the onset of AIDS and/or after the clinical appearance of AIDS.

- 5 Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP or related molecules. The effect of the test compound on AIDS is monitored according to standard methods. For example, analysis of the ability of the test compound to inhibit  
10 or prevent or ameliorate the destruction of helper T cells can be monitored. Comparative studies between treated and control animals are used to determine the relative efficacy of the test compounds in preventing or ameliorating AIDS. A molecule which prevents or  
15 ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of AIDS is considered useful in the invention.

In the invention also includes the use of a therapeutically effective amount rHuAFP (or fragment or  
20 analog thereof) for inhibiting the rejection of a transplanted organ (e.g., the heart, the liver, the lung, the pancreas, and the kidney), tissue (e.g., skin, bone marrow, dura mater, bone, implanted collagen, an implanted bioreactor), or cell (e.g.,  $\beta$  islet cells of  
25 the pancreas, stem cells, hematopoietic cells, lymph cells, neuroendocrine or adrenal cells) in a mammal. Such transplanted organs, tissues, or cells may be derived from any source, e.g., such biological material can be allogenic, phenogenic, autologous, synthetic,  
30 artificial or genetically-engineered. For example, the method can also be used when the patient is the recipient of an allograft such a heart or kidney from another species.

In one working example, the immunosuppressive  
35 effect of rHuAFP on clinical transplantation, i.e., the

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ability of rHuAFP to prevent or ameliorate transplant rejection (e.g., hyperacute rejection, acute rejection and chronic rejection), is evaluated by administering rHuAFP to an NIH minipig according to standard methods, e.g., intravenously or intraperitoneally, at an appropriate dosage on a daily basis. Generally, administration of rHuAFP is initiated prior to the transplant, e.g., transplantation of a kidney and/or after the transplant procedure. Control animals receive a placebo, e.g., human serum albumin, similarly administered as for rHuAFP. The effect of rHuAFP on transplant rejection is monitored according to standard methods. One manifestation of the rejection process is diminished function of the transplanted organ, for example, analysis of urine output can be monitored. If desired, histological inspection (e.g., by using any standard histochemical or immunohistochemical procedure, see e.g., Ausubel et al., supra; Bancroft and Stevens, supra) of kidney tissue is performed and tissue samples obtained by biopsy are examined microscopically for evidence of transplant rejection, e.g., chronic interstitial fibrosis, vascular thrombosis, or the presence of abnormal lymphocytic infiltrates. Comparative studies between treated and control animals are used to determine the relative efficacy of rHuAFP in preventing or ameliorating transplant rejection. Recombinant HuAFP (a fragment or analog thereof) which prevents or ameliorates (decreases or suppresses or relieves or promotes remission of) the symptoms of transplant rejection is considered useful in the invention.

All publications, manufacturer's instructions, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent

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application was specifically and individually indicated to be incorporated by reference.

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Murgita, Robert A.
- (ii) TITLE OF INVENTION: EXPRESSION AND PURIFICATION OF CLONED HUMAN ALPHA-FETOPROTEIN
- (iii) NUMBER OF SEQUENCES: 20
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Fish & Richardson P.C.
  - (B) STREET: 225 Franklin Street, Suite 3100
  - (C) CITY: Boston
  - (D) STATE: MA
  - (E) COUNTRY: USA
  - (F) ZIP: 02110-2804
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER: PCT/US96/----
  - (B) FILING DATE: 24-JAN-1996
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/377,317
  - (B) FILING DATE: 24-JAN-1995
  - (C) CLASSIFICATION:
  - (A) APPLICATION NUMBER: 08/377,311
  - (B) FILING DATE: 24-JAN-1995
  - (C) CLASSIFICATION:
  - (A) APPLICATION NUMBER: 08/377,309
  - (B) FILING DATE: 24-JAN-1995
  - (C) CLASSIFICATION:
  - (A) APPLICATION NUMBER: 08/377,316
  - (B) FILING DATE: 24-JAN-1995
  - (C) CLASSIFICATION:
  - (A) APPLICATION NUMBER: 08/505,012
  - (B) FILING DATE: 21-JULY-1995
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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  - (C) REFERENCE/DOCKET NUMBER: 06727/003001
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  - (B) TELEFAX: (617) 542-8906
  - (C) TELEX: 200154

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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGTCTGCAGG ATGGGGAAAA A

21

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CATGAAATGA CTCCAGTA

18

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 18 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATAGAAATG AATATGGA

18

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2022 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATATTGTGCT	TCCACCACTG	CCAATAACAA	AATAACTAGC	AACCATGAAG	TGGGTGGAAT	60
CAATTTTTTT	AATTTTCCTA	CTAAATTTTA	CTGAATCCAG	AACACTGCAT	AGAAATGAAT	120
ATGGAATAGC	TTCCATATTG	GATTCTTACC	AATGTACTGC	AGAGATAAGT	TTAGCTGACC	180
TGGCTACCAT	ATTTTTTGCC	CAGTTTGTTT	AAGAAGCCAC	TTACAAGGAA	GTAAGCAAAA	240
TGGTGAAAGA	TGCATTGACT	GCAATTGAGA	AACCCACTGG	AGATGAACAG	TCTTCAGGGT	300
GTTTAGAAAA	CCAGCTACCT	GCCTTTCTGG	AAGAAGTTTG	CCATGAGAAA	GAAATTTTGG	360
AGAAGTACGG	ACATTCAGAC	TGCTGCAGCC	AAAGTGAAGA	GGGAAGACAT	AACTGTTTTT	420
TTGCACACAA	AAAGCCCACT	GCAGCATGGA	TCCCACTTTT	CCAAGTTCCA	GAACCTGTCA	480
CAAGCTGTGA	AGCATATGAA	GAAGACAGGG	AGACATTCAT	GAACAAATTC	ATTTATGAGA	540
TAGCAAGAAG	GCATCCCTTC	CTGTATGCAC	CTACAATTCT	TCTTTCGGCT	GCTGGGTATG	600
AGAAAATAAT	TCCATCTTGC	TGCAAAGCTG	AAAATGCAGT	TGAATGCTTC	CAAACAAAGG	660
CAGCAACAGT	TACAAAAGAA	TTAAGAGAAA	GCAGCTTGTT	AAATCAACAT	GCATGTCCAG	720
TAATGAAAAA	TTTTGGGACC	CGAACTTTCC	AAGCCATAAC	TGTTACTAAA	CTGAGTCAGA	780
AGTTTACCAA	AGTTAATTTT	ACTGAAATCC	AGAACTAGT	CCTGGATGTG	GCCCATGTAC	840
ATGAGCACTG	TTGCAGAGCA	GATGTGCTGG	ATTGTCTGCA	GGATGGGGAA	AAAATCATGT	900
CCTACATATG	TTCTCAACAA	GACACTCTGT	CAAACAAAAT	AACAGAATGC	TGCAAACTGA	960
CCACGCTGGA	ACGTGGTCAA	TGTATAATTC	ATGCAGAAAA	TGATGAAAAA	CCTGAAGGTC	1020
TATCTCCAAA	TCTAAACAGG	TTTTTAGGAG	ATAGAGATTT	TAACCAATTT	TCTTCAGGGG	1080
AAAAAATAT	CTTCTTGCCA	AGTTTTGTTC	ATGAATATTC	AAGAAGACAT	CCTCAGCTTG	1140
CTGTCTCAGT	AATTCTAAGA	GTTGCTAAAG	GATACCAGGA	GTTATTGGAG	AAGTGTTCCT	1200
AGACTGAAAA	CCCTCTTGAA	TGCCAAGATA	AAGGAGAAGA	AGAATTACAG	AAATACATCC	1260
AGGAGAGCCA	AGCATTGGCA	AAGCGAAGCT	GCGGCCTCTT	CCAGAACTA	GGAGAATATT	1320
ACTTACAAAA	TGAGTTTCTC	GTTGCTTACA	CAAAGAAAGC	CCCCCAGCTG	ACCTCGTCGG	1380
AGCTGATGGC	CATCACCAGA	AAAATGGCAG	CCACAGCAGC	CACTTGTTGC	CAACTCAGTG	1440
AGGACAAACT	ATTGGCCTGT	GGCGAGGGAG	CGGCTGACAT	TATTATCGGA	CACTTATGTA	1500
TCAGACATGA	AATGACTCCA	GTAAACCCCTG	GTGTTGGCCA	GTGCTGCACT	TCTTCATATG	1560
CCAACAGGAG	GCCATGCTTC	AGCAGCTTGG	TGGTGGATGA	AACATATGTC	CCTCCTGCAT	1620
TCTCTGATGA	CAAGTTCATT	TTCCATAAGG	ATCTGTGCCA	AGCTCAGGGT	GTAGCGCTGC	1680
AAAGGATGAA	GCAAGAGTTT	CTCATTAACC	TTGTGAAGCA	AAAGCCACAA	ATAACAGAGG	1740
AACAACTTGA	GGCTCTCATT	GCAGATTTCT	CAGGCCTGTT	GGAGAAATGC	TGCCAAGGCC	1800
AGGAACAGGA	AGTCTGCTTT	GCTGAAGAGG	GACAAAAACT	GATTTCAAAA	ACTGGTGCTG	1860

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CTTTGGGAGT TTAAATTACT TCAGGGGAAG AGAAGACAAA ACGAGTCTTT CATTGGGTGT 1920  
 GAACTTTTCT CTTTAATTTT AACTGATTTA ACACTTTTTG TGAATTAATG ATAAAGACTT 1980  
 TTATGTGAGA TTTCCTTATC ACAGAAATAA AATATCTCCA AA 2022

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 590 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Thr Leu His Arg Asn Glu Tyr Gly Ile Ala Ser Ile Leu Asp Ser Tyr  
 1 5 10 15  
 Gln Cys Thr Ala Glu Ile Ser Leu Ala Asp Leu Ala Thr Ile Phe Phe  
 20 25 30  
 Ala Gln Phe Val Gln Glu Ala Thr Tyr Lys Glu Val Ser Lys Met Val  
 35 40 45  
 Lys Asp Ala Leu Thr Ala Ile Glu Lys Pro Thr Gly Asp Glu Gln Ser  
 50 55 60  
 Ser Gly Cys Leu Glu Asn Gln Leu Pro Ala Phe Leu Glu Glu Leu Cys  
 65 70 75 80  
 His Glu Lys Glu Ile Leu Glu Lys Tyr Gly His Ser Asp Cys Cys Ser  
 85 90 95  
 Gln Ser Glu Glu Gly Arg His Asn Cys Phe Leu Ala His Lys Lys Pro  
 100 105 110  
 Thr Ala Ala Trp Ile Pro Leu Phe Gln Val Pro Glu Pro Val Thr Ser  
 115 120 125  
 Cys Glu Ala Tyr Glu Glu Asp Arg Glu Thr Phe Met Asn Lys Phe Ile  
 130 135 140  
 Tyr Glu Ile Ala Arg Arg His Pro Phe Leu Tyr Ala Pro Thr Ile Leu  
 145 150 155 160  
 Leu Ser Ala Ala Gly Tyr Glu Lys Ile Ile Pro Ser Cys Cys Lys Ala  
 165 170 175  
 Glu Asn Ala Val Glu Cys Phe Gln Thr Lys Ala Ala Thr Val Thr Lys  
 180 185 190  
 Glu Leu Arg Glu Ser Ser Leu Leu Asn Gln His Ala Cys Pro Val Met  
 195 200 205  
 Lys Asn Phe Gly Thr Arg Thr Phe Gln Ala Ile Thr Val Thr Lys Leu  
 210 215 220



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Ser Gln Lys Phe Thr Lys Val Asn Phe Thr Glu Ile Gln Lys Leu Val  
 225 230 235 240  
 Leu Asp Val Ala His Val His Glu His Cys Cys Arg Ala Asp Val Leu  
 245 250 255  
 Asp Cys Leu Gln Asp Gly Glu Lys Ile Met Ser Tyr Ile Cys Ser Gln  
 260 265 270  
 Gln Asp Thr Leu Ser Asn Lys Ile Thr Glu Cys Cys Lys Leu Thr Thr  
 275 280 285  
 Leu Glu Arg Gly Gln Cys Ile Ile His Ala Glu Asn Asp Glu Lys Pro  
 290 295 300  
 Glu Gly Leu Ser Pro Asn Leu Asn Arg Phe Leu Gly Asp Arg Asp Phe  
 305 310 315 320  
 Asn Gln Phe Ser Ser Gly Glu Lys Asn Ile Phe Leu Ala Ser Phe Val  
 325 330 335  
 His Glu Tyr Ser Arg Arg His Pro Gln Leu Ala Val Ser Val Ile Leu  
 340 345 350  
 Arg Val Ala Lys Gly Tyr Gln Glu Leu Leu Glu Lys Cys Phe Gln Thr  
 355 360 365  
 Glu Asn Pro Leu Glu Cys Gln Asp Lys Gly Glu Glu Glu Leu Gln Lys  
 370 375 380  
 Tyr Ile Gln Glu Ser Gln Ala Leu Ala Lys Arg Ser Cys Gly Leu Phe  
 385 390 395 400  
 Gln Lys Leu Gly Glu Tyr Tyr Leu Gln Asn Glu Phe Leu Val Ala Tyr  
 405 410 415  
 Thr Lys Lys Ala Pro Gln Leu Thr Ser Ser Glu Leu Met Ala Ile Thr  
 420 425 430  
 Arg Lys Met Ala Ala Thr Ala Ala Thr Cys Cys Gln Leu Ser Glu Asp  
 435 440 445  
 Lys Leu Leu Ala Cys Gly Glu Gly Ala Ala Asp Ile Ile Ile Gly His  
 450 455 460  
 Leu Cys Ile Arg His Glu Met Thr Pro Val Asn Pro Gly Val Gly Gln  
 465 470 475 480  
 Cys Cys Thr Ser Ser Tyr Ala Asn Arg Arg Pro Cys Phe Ser Ser Leu  
 485 490 495  
 Val Val Asp Glu Thr Tyr Val Pro Pro Ala Phe Ser Asp Asp Lys Phe  
 500 505 510  
 Ile Phe His Lys Asp Leu Cys Gln Ala Gln Gly Val Ala Leu Gln Arg  
 515 520 525  
 Met Lys Gln Glu Phe Leu Ile Asn Leu Val Lys Gln Lys Pro Gln Ile  
 530 535 540  
 Thr Glu Glu Gln Leu Glu Ala Leu Ile Ala Asp Phe Ser Gly Leu Leu  
 545 550 555 560

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Glu Lys Cys Cys Gln Gly Gln Glu Gln Glu Val Cys Phe Ala Glu Glu  
565 570 575

Gly Gln Lys Leu Ile Ser Lys Thr Gly Ala Ala Leu Gly Val  
580 585 590

## (2) INFORMATION FOR SEQ ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 197 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Thr Leu His Arg Asn Glu Tyr Gly Ile Ala Ser Ile Leu Asp Ser Tyr  
1 5 10  
Gln Cys Thr Ala Glu Ile Ser Leu Ala Asp Leu Ala Thr Ile Phe Phe  
20 25 30  
Ala Gln Phe Val Gln Glu Ala Thr Tyr Lys Glu Val Ser Lys Met Val  
35 40 45  
Lys Asp Ala Leu Thr Ala Ile Glu Lys Pro Thr Gly Asp Glu Gln Ser  
50 55 60  
Ser Gly Cys Leu Glu Asn Gln Leu Pro Ala Phe Leu Glu Glu Leu Cys  
65 70 75 80  
His Glu Lys Glu Ile Leu Glu Lys Tyr Gly His Ser Asp Cys Cys Ser  
85 90 95  
Gln Ser Glu Glu Gly Arg His Asn Cys Phe Leu Ala His Lys Lys Pro  
100 105 110  
Thr Ala Ala Trp Ile Pro Leu Phe Gln Val Pro Glu Pro Val Thr Ser  
115 120 125  
Cys Glu Ala Tyr Glu Glu Asp Arg Glu Thr Phe Met Asn Lys Phe Ile  
130 135 140  
Tyr Glu Ile Ala Arg Arg His Pro Phe Leu Tyr Ala Pro Thr Ile Leu  
145 150 155 160  
Leu Ser Ala Ala Gly Tyr Glu Lys Ile Ile Pro Ser Cys Cys Lys Ala  
165 170 175  
Glu Asn Ala Val Glu Cys Phe Gln Thr Lys Ala Ala Thr Val Thr Lys  
180 185 190  
Glu Leu Arg Glu Ser  
195

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 192 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

Ser Leu Leu Asn Gln His Ala Cys Pro Val Met Lys Asn Phe Gly Thr
 1              5              10              15
Arg Thr Phe Gln Ala Ile Thr Val Thr Lys Leu Ser Gln Lys Phe Thr
      20              25              30
Lys Val Asn Phe Thr Glu Ile Gln Lys Leu Val Leu Asp Val Ala His
      35              40              45
Val His Glu His Cys Cys Arg Ala Asp Val Leu Asp Cys Leu Gln Asp
      50              55              60
Gly Glu Lys Ile Met Ser Tyr Ile Cys Ser Gln Gln Asp Thr Leu Ser
      65              70              75              80
Asn Lys Ile Thr Glu Cys Cys Lys Leu Thr Thr Leu Glu Arg Gly Gln
      85              90              95
Cys Ile Ile His Ala Glu Asn Asp Glu Lys Pro Glu Gly Leu Ser Pro
      100             105             110
Asn Leu Asn Arg Phe Leu Gly Asp Arg Asp Phe Asn Gln Phe Ser Ser
      115             120             125
Gly Glu Lys Asn Ile Phe Leu Ala Ser Phe Val His Glu Tyr Ser Arg
      130             135             140
Arg His Pro Gln Leu Ala Val Ser Val Ile Leu Arg Val Ala Lys Gly
      145             150             155             160
Tyr Gln Glu Leu Leu Glu Lys Cys Phe Gln Thr Glu Asn Pro Leu Glu
      165             170             175
Cys Gln Asp Lys Gly Glu Glu Glu Leu Gln Lys Tyr Ile Gln Glu Ser
      180             185             190

```

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 201 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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Gln Ala Leu Ala Lys Arg Ser Cys Gly Leu Phe Gln Lys Leu Gly Glu  
 1 5 10 15  
 Tyr Tyr Leu Gln Asn Glu Phe Leu Val Ala Tyr Thr Lys Lys Ala Pro  
 20 25 30  
 Gln Leu Thr Ser Ser Glu Leu Met Ala Ile Thr Arg Lys Met Ala Ala  
 35 40 45  
 Thr Ala Ala Thr Cys Cys Gln Leu Ser Glu Asp Lys Leu Leu Ala Cys  
 50 55 60  
 Gly Glu Gly Ala Ala Asp Ile Ile Ile Gly His Leu Cys Ile Arg His  
 65 70 75 80  
 Glu Met Thr Pro Val Asn Pro Gly Val Gly Gln Cys Cys Thr Ser Ser  
 85 90 95  
 Tyr Ala Asn Arg Arg Pro Cys Phe Ser Ser Leu Val Val Asp Glu Thr  
 100 105 110  
 Tyr Val Pro Pro Ala Phe Ser Asp Asp Lys Phe Ile Phe His Lys Asp  
 115 120 125  
 Leu Cys Gln Ala Gln Gly Val Ala Leu Gln Arg Met Lys Gln Glu Phe  
 130 135 140  
 Leu Ile Asn Leu Val Lys Gln Lys Pro Gln Ile Thr Glu Glu Gln Leu  
 145 150 155 160  
 Glu Ala Leu Ile Ala Asp Phe Ser Gly Leu Leu Glu Lys Cys Cys Gln  
 165 170 175  
 Gly Gln Glu Gln Glu Val Cys Phe Ala Glu Glu Gly Gln Lys Leu Ile  
 180 185 190  
 Ser Lys Thr Gly Ala Ala Leu Gly Val  
 195 200

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 389 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Thr Leu His Arg Asn Glu Tyr Gly Ile Ala Ser Ile Leu Asp Ser Tyr  
 1 5 10 15  
 Gln Cys Thr Ala Glu Ile Ser Leu Ala Asp Leu Ala Thr Ile Phe Phe  
 20 25 30  
 Ala Gln Phe Val Gln Glu Ala Thr Tyr Lys Glu Val Ser Lys Met Val  
 35 40 45

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Lys Asp Ala Leu Thr Ala Ile Glu Lys Pro Thr Gly Asp Glu Gln Ser  
 50 55 60  
 Ser Gly Cys Leu Glu Asn Gln Leu Pro Ala Phe Leu Glu Glu Leu Cys  
 65 70 75 80  
 His Glu Lys Glu Ile Leu Glu Lys Tyr Gly His Ser Asp Cys Cys Ser  
 85 90 95  
 Gln Ser Glu Glu Gly Arg His Asn Cys Phe Leu Ala His Lys Lys Pro  
 100 105 110  
 Thr Ala Ala Trp Ile Pro Leu Phe Gln Val Pro Glu Pro Val Thr Ser  
 115 120 125  
 Cys Glu Ala Tyr Glu Glu Asp Arg Glu Thr Phe Met Asn Lys Phe Ile  
 130 135 140  
 Tyr Glu Ile Ala Arg Arg His Pro Phe Leu Tyr Ala Pro Thr Ile Leu  
 145 150 155 160  
 Leu Ser Ala Ala Gly Tyr Glu Lys Ile Ile Pro Ser Cys Cys Lys Ala  
 165 170 175  
 Glu Asn Ala Val Glu Cys Phe Gln Thr Lys Ala Ala Thr Val Thr Lys  
 180 185 190  
 Glu Leu Arg Glu Ser Ser Leu Leu Asn Gln His Ala Cys Pro Val Met  
 195 200 205  
 Lys Asn Phe Gly Thr Arg Thr Phe Gln Ala Ile Thr Val Thr Lys Leu  
 210 215 220  
 Ser Gln Lys Phe Thr Lys Val Asn Phe Thr Glu Ile Gln Lys Leu Val  
 225 230 235 240  
 Leu Asp Val Ala His Val His Glu His Cys Cys Arg Ala Asp Val Leu  
 245 250 255  
 Asp Cys Leu Gln Asp Gly Glu Lys Ile Met Ser Tyr Ile Cys Ser Gln  
 260 265 270  
 Gln Asp Thr Leu Ser Asn Lys Ile Thr Glu Cys Cys Lys Leu Thr Thr  
 275 280 285  
 Leu Glu Arg Gly Gln Cys Ile Ile His Ala Glu Asn Asp Glu Lys Pro  
 290 295 300  
 Glu Gly Leu Ser Pro Asn Leu Asn Arg Phe Leu Gly Asp Arg Asp Phe  
 305 310 315 320  
 Asn Gln Phe Ser Ser Gly Glu Lys Asn Ile Phe Leu Ala Ser Phe Val  
 325 330 335  
 His Glu Tyr Ser Arg Arg His Pro Gln Leu Ala Val Ser Val Ile Leu  
 340 345 350  
 Arg Val Ala Lys Gly Tyr Gln Glu Leu Leu Glu Lys Cys Phe Gln Thr  
 355 360 365  
 Glu Asn Pro Leu Glu Cys Gln Asp Lys Gly Glu Glu Glu Leu Gln Lys  
 370 375 380

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Tyr Ile Gln Glu Ser  
385

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 393 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ser	Leu	Leu	Asn	Gln	His	Ala	Cys	Pro	Val	Met	Lys	Asn	Phe	Gly	Thr	1	5	10	15
Arg	Thr	Phe	Gln	Ala	Ile	Thr	Val	Thr	Lys	Leu	Ser	Gln	Lys	Phe	Thr	20	25	30	
Lys	Val	Asn	Phe	Thr	Glu	Ile	Gln	Lys	Leu	Val	Leu	Asp	Val	Ala	His	35	40	45	
Val	His	Glu	His	Cys	Cys	Arg	Ala	Asp	Val	Leu	Asp	Cys	Leu	Gln	Asp	50	55	60	
Gly	Glu	Lys	Ile	Met	Ser	Tyr	Ile	Cys	Ser	Gln	Gln	Asp	Thr	Leu	Ser	65	70	75	80
Asn	Lys	Ile	Thr	Glu	Cys	Cys	Lys	Leu	Thr	Thr	Leu	Glu	Arg	Gly	Gln	85	90	95	
Cys	Ile	Ile	His	Ala	Glu	Asn	Asp	Glu	Lys	Pro	Glu	Gly	Leu	Ser	Pro	100	105	110	
Asn	Leu	Asn	Arg	Phe	Leu	Gly	Asp	Arg	Asp	Phe	Asn	Gln	Phe	Ser	Ser	115	120	125	
Gly	Glu	Lys	Asn	Ile	Phe	Leu	Ala	Ser	Phe	Val	His	Glu	Tyr	Ser	Arg	130	135	140	
Arg	His	Pro	Gln	Leu	Ala	Val	Ser	Val	Ile	Leu	Arg	Val	Ala	Lys	Gly	145	150	155	160
Tyr	Gln	Glu	Leu	Leu	Glu	Lys	Cys	Phe	Gln	Thr	Glu	Asn	Pro	Leu	Glu	165	170	175	
Cys	Gln	Asp	Lys	Gly	Glu	Glu	Glu	Leu	Gln	Lys	Tyr	Ile	Gln	Glu	Ser	180	185	190	
Gln	Ala	Leu	Ala	Lys	Arg	Ser	Cys	Gly	Leu	Phe	Gln	Lys	Leu	Gly	Glu	195	200	205	
Tyr	Tyr	Leu	Gln	Asn	Glu	Phe	Leu	Val	Ala	Tyr	Thr	Lys	Lys	Ala	Pro	210	215	220	
Gln	Leu	Thr	Ser	Ser	Glu	Leu	Met	Ala	Ile	Thr	Arg	Lys	Met	Ala	Ala	225	230	235	240

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Thr	Ala	Ala	Thr	Cys 245	Cys	Gln	Leu	Ser	Glu 250	Asp	Lys	Leu	Leu	Ala 255	Cys
Gly	Glu	Gly	Ala 260	Ala	Asp	Ile	Ile	Ile 265	Gly	His	Leu	Cys	Ile 270	Arg	His
Glu	Met	Thr 275	Pro	Val	Asn	Pro	Gly 280	Val	Gly	Gln	Cys	Cys 285	Thr	Ser	Ser
Tyr	Ala 290	Asn	Arg	Arg	Pro	Cys 295	Phe	Ser	Ser	Leu	Val 300	Val	Asp	Glu	Thr
Tyr 305	Val	Pro	Pro	Ala	Phe 310	Ser	Asp	Asp	Lys	Phe 315	Ile	Phe	His	Lys	Asp 320
Leu	Cys	Gln	Ala	Gln 325	Gly	Val	Ala	Leu	Gln 330	Arg	Met	Lys	Gln	Glu 335	Phe
Leu	Ile	Asn	Leu 340	Val	Lys	Gln	Lys	Pro 345	Gln	Ile	Thr	Glu	Glu 350	Gln	Leu
Glu	Ala	Leu 355	Ile	Ala	Asp	Phe	Ser 360	Gly	Leu	Leu	Glu	Lys 365	Cys	Cys	Gln
Gly	Gln 370	Glu	Gln	Glu	Val	Cys 375	Phe	Ala	Glu	Glu	Gly 380	Gln	Lys	Leu	Ile
Ser 385	Lys	Thr	Gly	Ala	Ala 390	Leu	Gly	Val							

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 325 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: not relevant
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met 1	Ser	Tyr	Ile 5	Cys	Ser	Gln	Gln	Asp 10	Thr	Leu	Ser	Asn	Lys	Ile 15	Thr
Glu	Cys	Cys 20	Lys	Leu	Thr	Thr	Leu	Glu 25	Arg	Gly	Gln	Cys	Ile 30	Ile	His
Ala	Glu	Asn 35	Asp	Glu	Lys	Pro	Glu 40	Gly	Leu	Ser	Pro	Asn 45	Leu	Asn	Arg
Phe 50	Leu	Gly	Asp	Arg	Asp	Phe 55	Asn	Gln	Phe	Ser	Ser 60	Gly	Glu	Lys	Asn
Ile 65	Phe	Leu	Ala	Ser	Phe 70	Val	His	Glu	Tyr	Ser 75	Arg	Arg	His	Pro	Gln 80
Leu	Ala	Val	Ser 85	Val	Ile	Leu	Arg	Val 90	Ala	Lys	Gly	Tyr	Gln	Glu 95	Leu

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Leu Glu Lys Cys Phe Gln Thr Glu Asn Pro Leu Glu Cys Gln Asp Lys  
 100 105 110  
 Gly Glu Glu Glu Leu Gln Lys Tyr Ile Gln Glu Ser Gln Ala Leu Ala  
 115 120 125  
 Lys Arg Ser Cys Gly Leu Phe Gln Lys Leu Gly Glu Tyr Tyr Leu Gln  
 130 135 140  
 Asn Glu Phe Leu Val Ala Tyr Thr Lys Lys Ala Pro Gln Leu Thr Ser  
 145 150 155 160  
 Ser Glu Leu Met Ala Ile Thr Arg Lys Met Ala Ala Thr Ala Ala Thr  
 165 170 175  
 Cys Cys Gln Leu Ser Glu Asp Lys Leu Leu Ala Cys Gly Glu Gly Ala  
 180 185 190  
 Ala Asp Ile Ile Ile Gly His Leu Cys Ile Arg His Glu Met Thr Pro  
 195 200 205  
 Val Asn Pro Gly Val Gly Gln Cys Cys Thr Ser Ser Tyr Ala Asn Arg  
 210 215 220  
 Arg Pro Cys Phe Ser Ser Leu Val Val Asp Glu Thr Tyr Val Pro Pro  
 225 230 235 240  
 Ala Phe Ser Asp Asp Lys Phe Ile Phe His Lys Asp Leu Cys Gln Ala  
 245 250 255  
 Gln Gly Val Ala Leu Gln Arg Met Lys Gln Glu Phe Leu Ile Asn Leu  
 260 265 270  
 Val Lys Gln Lys Pro Gln Ile Thr Glu Glu Gln Leu Glu Ala Leu Ile  
 275 280 285  
 Ala Asp Phe Ser Gly Leu Leu Glu Lys Cys Cys Gln Gly Gln Glu Gln  
 290 295 300  
 Glu Val Cys Phe Ala Glu Glu Gly Gln Lys Leu Ile Ser Lys Thr Gly  
 305 310 315 320  
 Ala Ala Leu Gly Val  
 325

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 324 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ser Tyr Ile Cys Ser Gln Gln Asp Thr Leu Ser Asn Lys Ile Thr Glu  
 1 5 10 15



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Cys Cys Lys Leu Thr Thr Leu Glu Arg Gly Gln Cys Ile Ile His Ala  
                     20                    25                    30  
 Glu Asn Asp Glu Lys Pro Glu Gly Leu Ser Pro Asn Leu Asn Arg Phe  
                     35                    40                    45  
 Leu Gly Asp Arg Asp Phe Asn Gln Phe Ser Ser Gly Glu Lys Asn Ile  
                     50                    55                    60  
 Phe Leu Ala Ser Phe Val His Glu Tyr Ser Arg Arg His Pro Gln Leu  
                     65                    70                    75                    80  
 Ala Val Ser Val Ile Leu Arg Val Ala Lys Gly Tyr Gln Glu Leu Leu  
                     85                    90                    95  
 Glu Lys Cys Phe Gln Thr Glu Asn Pro Leu Glu Cys Gln Asp Lys Gly  
                     100                    105                    110  
 Glu Glu Glu Leu Gln Lys Tyr Ile Gln Glu Ser Gln Ala Leu Ala Lys  
                     115                    120                    125  
 Arg Ser Cys Gly Leu Phe Gln Lys Leu Gly Glu Tyr Tyr Leu Gln Asn  
                     130                    135                    140  
 Glu Phe Leu Val Ala Tyr Thr Lys Lys Ala Pro Gln Leu Thr Ser Ser  
                     145                    150                    155                    160  
 Glu Leu Met Ala Ile Thr Arg Lys Met Ala Ala Thr Ala Ala Thr Cys  
                     165                    170                    175  
 Cys Gln Leu Ser Glu Asp Lys Leu Leu Ala Cys Gly Glu Gly Ala Ala  
                     180                    185                    190  
 Asp Ile Ile Ile Gly His Leu Cys Ile Arg His Glu Met Thr Pro Val  
                     195                    200                    205  
 Asn Pro Gly Val Gly Gln Cys Cys Thr Ser Ser Tyr Ala Asn Arg Arg  
                     210                    215                    220  
 Pro Cys Phe Ser Ser Leu Val Val Asp Glu Thr Tyr Val Pro Pro Ala  
                     225                    230                    235                    240  
 Phe Ser Asp Asp Lys Phe Ile Phe His Lys Asp Leu Cys Gln Ala Gln  
                     245                    250                    255  
 Gly Val Ala Leu Gln Arg Met Lys Gln Glu Phe Leu Ile Asn Leu Val  
                     260                    265                    270  
 Lys Gln Lys Pro Gln Ile Thr Glu Glu Gln Leu Glu Ala Leu Ile Ala  
                     275                    280                    285  
 Asp Phe Ser Gly Leu Leu Glu Lys Cys Cys Gln Gly Gln Glu Gln Glu  
                     290                    295                    300  
 Val Cys Phe Ala Glu Glu Gly Gln Lys Leu Ile Ser Lys Thr Gly Ala  
                     305                    310                    315                    320  
 Ala Leu Gly Val

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

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- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ser Tyr Ile Cys Ser Gln Gln Asp Thr  
1 5

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 30 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AAAAAAGGTA CCACACTGCA TAGAAATGAA

30

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAAAAAGGAT CCTTAGCTTT CTCTTAATTC TTT

33

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

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AAAAAATCG ATATGAGCTT GTTAAATCAA CAT

33

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AAAAAAGGAT CCTTAGCTCT CCTGGATGTA TTT

33

## (2) INFORMATION FOR SEQ ID NO:18:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAAAAATCG ATATGCAAGC ATTGGCAAAG CGA

33

## (2) INFORMATION FOR SEQ ID NO:19:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAAAAAGGAT CCTTAACTC CCAAAGCAGC ACG

33

## (2) INFORMATION FOR SEQ ID NO:20:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAAAAAATCG ATATGTCCTA CATATGTTCT CAA

33

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GATCTAGAAT TCGGATCCGG T

21

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: not relevant
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Asp	Leu	Glu	Phe	Met	Thr	Leu	His	Arg	Asn
1				5					10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AAAAAACTCG AGATACTG CATAGAAATG AA

32

(2) INFORMATION FOR SEQ ID NO:24:

- 103 -

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AAAAAAGAAT TCTTAAACTC CCAAAGCAGC ACG

33

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I claim:

- 105 -

Claims

1. Substantially pure biologically-active  
recombinant human alpha-fetoprotein comprising a sequence  
substantially identical to amino acids 1 to 389 of Fig. 1  
5 (SEQ ID NO: 9) or a fragment thereof.

2. The pure recombinant human alpha-fetoprotein  
of claim 1, wherein said human alpha-fetoprotein is  
produced using a prokaryotic cell.

3. Substantially pure biologically-active  
10 recombinant human alpha-fetoprotein comprising a sequence  
substantially identical to amino acids 198 to 590 of Fig.  
1 (SEQ ID NO: 10) or a fragment thereof.

4. The pure recombinant human alpha-fetoprotein  
of claim 3, wherein said human alpha-fetoprotein is  
15 produced using a prokaryotic cell.

5. Substantially pure biologically-active  
recombinant human alpha-fetoprotein comprising a sequence  
substantially identical to amino acids 198 to 389 of Fig.  
1 (SEQ ID NO: 7) or a fragment thereof.

20 6. The pure recombinant human alpha-fetoprotein  
of claim 5, wherein said human alpha-fetoprotein is  
produced using a prokaryotic cell.

7. Substantially pure biologically active  
recombinant human alpha-fetoprotein comprising a sequence  
25 substantially identical to amino acids 390 to 590 of Fig.  
1 (SEQ ID NO: 8) or a fragment thereof.

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8. The pure recombinant human alpha-fetoprotein of claim 7, wherein said human alpha-fetoprotein is produced using a prokaryotic cell.

9. Substantially pure biologically active  
5 recombinant human alpha-fetoprotein comprising a sequence substantially identical to amino acids 267 to 590 of Fig. 1 (SEQ ID NO: 11) or a fragment thereof.

10. The pure recombinant human alpha-fetoprotein of claim 9, wherein said human alpha-fetoprotein is  
10 produced using a prokaryotic cell.

11. A therapeutic composition comprising the substantially pure human recombinant alpha-fetoprotein of claims 1, 3, 5, 7, and 9.

12. A method for using an insect cell for  
15 producing biologically active recombinant human alpha-fetoprotein or a fragment or analog thereof comprising  
a) providing a transformed insect cell comprising a recombinant DNA molecule encoding said human alpha-fetoprotein or fragment or analog thereof operably linked  
20 to an expression control element which directs the expression of said human alpha-fetoprotein or fragment or analog thereof;

b) culturing said transformed cell; and

c) recovering said biologically active human  
25 alpha-fetoprotein or fragment or analog thereof.

13. The method of claim 12, wherein said insect cell is *Spodoptera frugiperda*.



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14. Substantially pure human alpha-fetoprotein or fragment or analog thereof produced by the method of claim 12.

15. A therapeutic composition comprising the  
5 substantially pure human alpha-fetoprotein or fragment or analog thereof of claim 14.

16. A method of inhibiting autoreactive immune cell proliferation in a mammal, said method comprising administering to said mammal a therapeutically effective  
10 amount of recombinant human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof.

17. The method of claim 16, wherein said immune cells include T cells.

15 18. The method of claim 16, wherein said immune cells include B cells.

19. The method of claim 16, wherein said mammal is a human patient.

20. The method of claim 16, wherein said  
20 recombinant alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

21. The method of claim 20, wherein said prokaryotic cell is E. coli.

22. A method of treating an autoimmune disease in  
25 a mammal, said method comprising administering to said mammal a therapeutically effective amount of recombinant

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human alpha-fetoprotein or an immune cell anti-proliferative fragment or analog thereof.

23. The method of claim 22, wherein said autoimmune disease is multiple sclerosis.

5           24. The method of claim 22, wherein said autoimmune disease is rheumatoid arthritis.

25. The method of claim 22, wherein said autoimmune disease is myasthenia gravis.

26. The method of claim 22, wherein said  
10 autoimmune disease is insulin-dependent diabetes mellitus.

27. The method of claim 22 wherein said autoimmune disease is systemic lupus erythematosus.

28. The method of claim 22, wherein said  
15 recombinant alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

29. The method of claim 28, wherein said prokaryotic cell is E. coli.

30. The method of claim 16, further comprising  
20 administering to said mammal an immunosuppressive agent in an effective dose which is lower than the standard dose when said immunosuppressive agent is used by itself.

31. The method of claim 16, further comprising administering to said mammal a tolerizing agent.

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32. The method of claim 30 or claim 31, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

33. The method of claim 32, wherein said  
5 prokaryotic cell is E. coli.

34. The method of claim 30, wherein said immunosuppressive agent is cyclosporine.

35. The method of claim 30, wherein said immunosuppressive agent is a steroid, azathioprine, FK-  
10 506, or 15-deoxyspergualin.

36. A method of inhibiting a neoplasm in a mammal, said method comprising administering to said mammal a therapeutically effective amount of recombinant human alpha-fetoprotein or an anti-neoplasm fragment or  
15 analog thereof.

37. The method of claim 36, wherein said mammal is a human patient.

38. The method of claim 36, wherein said neoplasm is a malignant tumor.

20 39. The method claim 38, wherein said malignant tumor is a breast tumor.

40. The method of claim 38, wherein said malignant tumor is a prostate tumor.

41. The method of claim 36, wherein said  
25 recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

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42. The method of claim 41, wherein said prokaryotic cell is E. coli.

43. The method of claim 36, wherein cells of said neoplasm express a receptor which is recognized by said  
5 recombinant human alpha-fetoprotein.

44. The method of claim 36, wherein said neoplasm is a carcinoma.

45. The method of claim 36, wherein said neoplasm is an adenocarcinoma.

10 46. The method of claim 36, wherein said neoplasm is a sarcoma.

47. The method of claim 36, wherein said neoplasm proliferates in response to estrogen.

15 48. The method of claim 36, wherein said administering inhibits proliferation of cells of said neoplasm in said mammal.

49. The method of claim 36, wherein said administering kills cells of said neoplasm in said mammal.

20 50. The method of claim 36, further comprising administering to said mammal a chemotherapeutic agent in an effective dose which is lower than the standard dose when said chemotherapeutic agent is used by itself.

51. A method of protecting a mammal from  
25 developing a neoplasm, comprising administering to said

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mammal a therapeutically effective amount of recombinant human alpha-fetoprotein.

52. The method of claim 51, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

53. The method of claim 52, wherein said prokaryotic cell is E. coli.

54. A hybrid cytotoxin comprising recombinant human alpha-fetoprotein linked to a cytotoxic agent.

55. The hybrid cytotoxin of claim 54, wherein said cytotoxic agent is a protein.

56. The hybrid cytotoxin of claim 54, wherein said cytotoxic agent is chemically conjugated to said recombinant human alpha-fetoprotein.

57. The hybrid cytotoxin of claim 54, wherein said cytotoxin is linked by a peptide bond to said recombinant human alpha-fetoprotein, and said hybrid toxin is produced by expression of a genetically engineered hybrid DNA molecule.

58. A detectably-labelled recombinant human alpha-fetoprotein or a detectably-labelled fragment or analog thereof capable of binding to a human neoplastic cell.

59. The molecule of claim 58 labelled with a radionuclide.

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60. The molecule of claim 59, wherein said radionuclide is technetium-99m.

61. The molecule of claim 58, wherein said recombinant human alpha-fetoprotein is produced in a  
5 prokaryotic cell and is unglycosylated.

62. The molecule of claim 61, wherein said prokaryotic cell is E. coli.

63. A method of imaging a neoplastic cell-containing region in a human patient in vivo, said method  
10 comprising:

- (a) providing a detectably-labelled molecule of claim 58;
- (b) administering said molecule to said patient;
- (c) allowing said labelled molecule to bind and  
15 allowing unbound molecule to be cleared from said region containing said region; and
- (d) obtaining an image of said neoplastic cell-containing region.

64. The method of claim 63, wherein said region  
20 is the breast.

65. The method of claim 63, wherein said region is the prostate.

66. The method of claim 63, wherein said region is bone marrow.

25 67. The method of claim 63, wherein said region is the liver.

- 113 -

68. The method of claim 63, wherein said image is obtained using scintigraphy.

69. A method for diagnosing a neoplasm in a biological sample, said method comprising:

- 5 (a) contacting said biological sample with the detectably-labelled molecule of claim 58; and  
(b) detecting said label bound to said sample, wherein the detection of label above background levels is indicative that said patient has a neoplasm.

10 70. The method of claim 69, wherein said biological sample comprises cells fixed and sectioned prior to said contacting step, and said label bound to said sample is bound to areas corresponding to the cell membrane of said cells.

15 71. The method of claim 69, wherein said biological sample is from the breast of a human patient.

72. The method of claim 69, wherein said biological sample is from the prostate of a human patient.

20 73. A method of detecting a neoplasm a mammal in vivo, comprising:

- (a) administering a diagnostically effective amount of the detectably-labelled molecule of claim 58; and  
25 (c) detecting the presence of said detectable label bound to a tissue of said mammal, wherein an amount of label above background levels is indicative of the presence of said neoplasm in said mammal.

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74. The method of claim 73, wherein said mammal is a human patient suspected of having a breast cancer, and said tissue is breast tissue.

75. The method of claim 73, wherein said mammal  
5 is a human patient suspected of having a prostate cancer, and said tissue is prostate tissue.

76. The method of claim 73, wherein said detectable label is a radionuclide and said detection step is accomplished by radioimaging.

10 77. The method of claim 76, wherein said radionuclide is technetium-99m and said radioimaging is scintigraphy.

78. A kit comprising  
(a) a first reagent comprising recombinant human  
15 alpha-fetoprotein or a fragment or analog thereof; and  
(b) a second reagent comprising a detectable label.

79. The kit of claim 78, wherein said detectable label is a radionuclide.

20 80. The kit of claim 79, wherein said radionuclide is technetium-99m.

81. The kit of claim 79, wherein said radionuclide includes iodine or indium.

82. The kit of claim 78, wherein said kit further  
25 includes a third reagent for linking said detectable label to said recombinant human alpha-fetoprotein or a fragment or analog thereof.



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83. The kit of claim 78, wherein said detectable label includes an enzyme, a flurophore, or an antibody.

84. The kit of claim 78, wherein said kit further includes a fourth reagent for detecting said detectable  
5 label linked to said recombinant human alpha-fetoprotein or a fragment or analog thereof.

85. The kit of claim 78, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

10 86. The kit of claim 78, wherein said prokaryotic cell is E. coli.

87. A cell culture medium comprising recombinant human alpha-fetoprotein or a cell-stimulating fragment or analog thereof.

15 88. The medium of claim 87, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

89. The media of claim 88, wherein said prokaryotic cell is E. coli.

20 90. A method of cell culture, said method comprising (a) providing the culture medium of claim 87; (b) providing a cell; and (c) growing said cell in said medium, wherein said cell proliferates, and is maintained.

25 91. The method of claim 90, wherein said cell is a mammalian cell.

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92. The method of claim 91, wherein said cell is a bone marrow cell.

93. The method of claim 92, wherein said bone marrow cell is a T cells.

5           94. The method of claim 92, wherein said bone marrow cell is a natural killer cell.

95. The method of claim 92, wherein said bone marrow cell is a lymphocyte.

96. The method of claim 91, wherein said cell is  
10 a hybridoma.

97. The method of claim 90, wherein said method involves ex vivo cell culture.

98. A method for inhibiting myelotoxycity in a mammal comprising administering to said mammal a  
15 therapeutically effective amount of recombinant human alpha-fetoprotein or a myelotoxic-inhibiting analog or fragment thereof.

99. The method of claim 98, wherein said mammal is a human patient.

20           100. The method of claim 99, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

101. The method of claim 100, wherein said prokaryotic cell is E. coli.

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102. A method of inhibiting suppression of bone marrow cell proliferation in a mammal, said method comprising administering to said mammal an effective amount of recombinant alpha-fetoprotein or an anti-  
5 suppressive fragment or analog thereof.

103. The method of claim 102, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

104. The method of claim 103, wherein said  
10 prokaryotic cell is E. coli.

105. A method of promoting bone marrow cell proliferation in a mammal, said method comprising administering to said mammal an effective amount of recombinant human alpha-fetoprotein or a cell-stimulating  
15 fragment or analog thereof.

106. The method of claim 105, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

107. The method of claim 106, wherein said  
20 prokaryotic cell is E. coli.

108. A method of preventing bone marrow cell transplantation rejection in a mammal, said method comprising administering to said mammal an effective amount of recombinant human alpha-fetoprotein or an anti-  
25 rejection fragment or analog thereof.

109. The method of claim 108, wherein said recombinant human alpha-fetoprotein is produced in a prokaryotic cell and is unglycosylated.

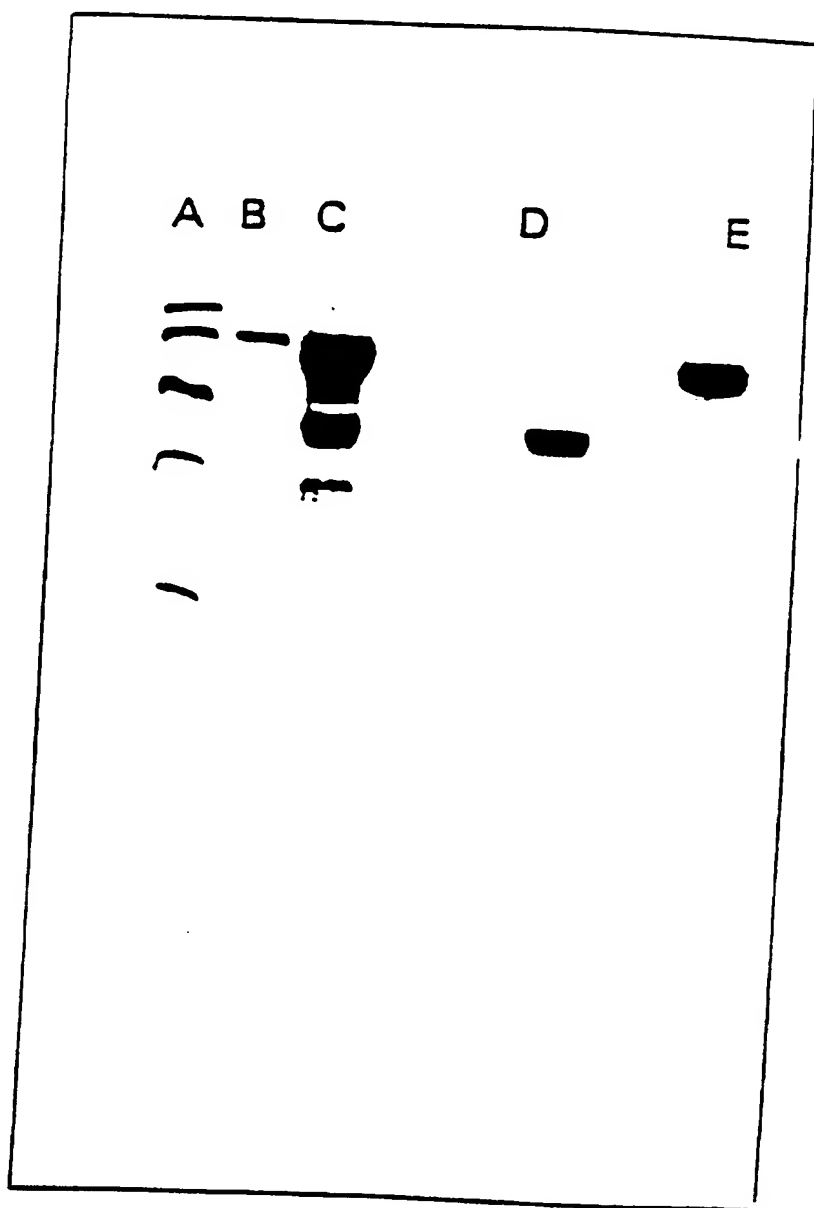
- 118 -

110. The method of claim 109, wherein said prokaryotic cell is E. coli.

TTGATCGGTGTGAAGCTTTCTCTTTAAATTTAACTGATTTAAGCTTTTGTGATTTAAATGAATCATAAAGCTTTTATGTGAAATTTCTTATCAGACAAATAAAAATCTCGAAAT 3071

2/10

Figure 2



# Inhibition of the Human AMLR by E.coli - Derived rHuAFP and Domain Fragments

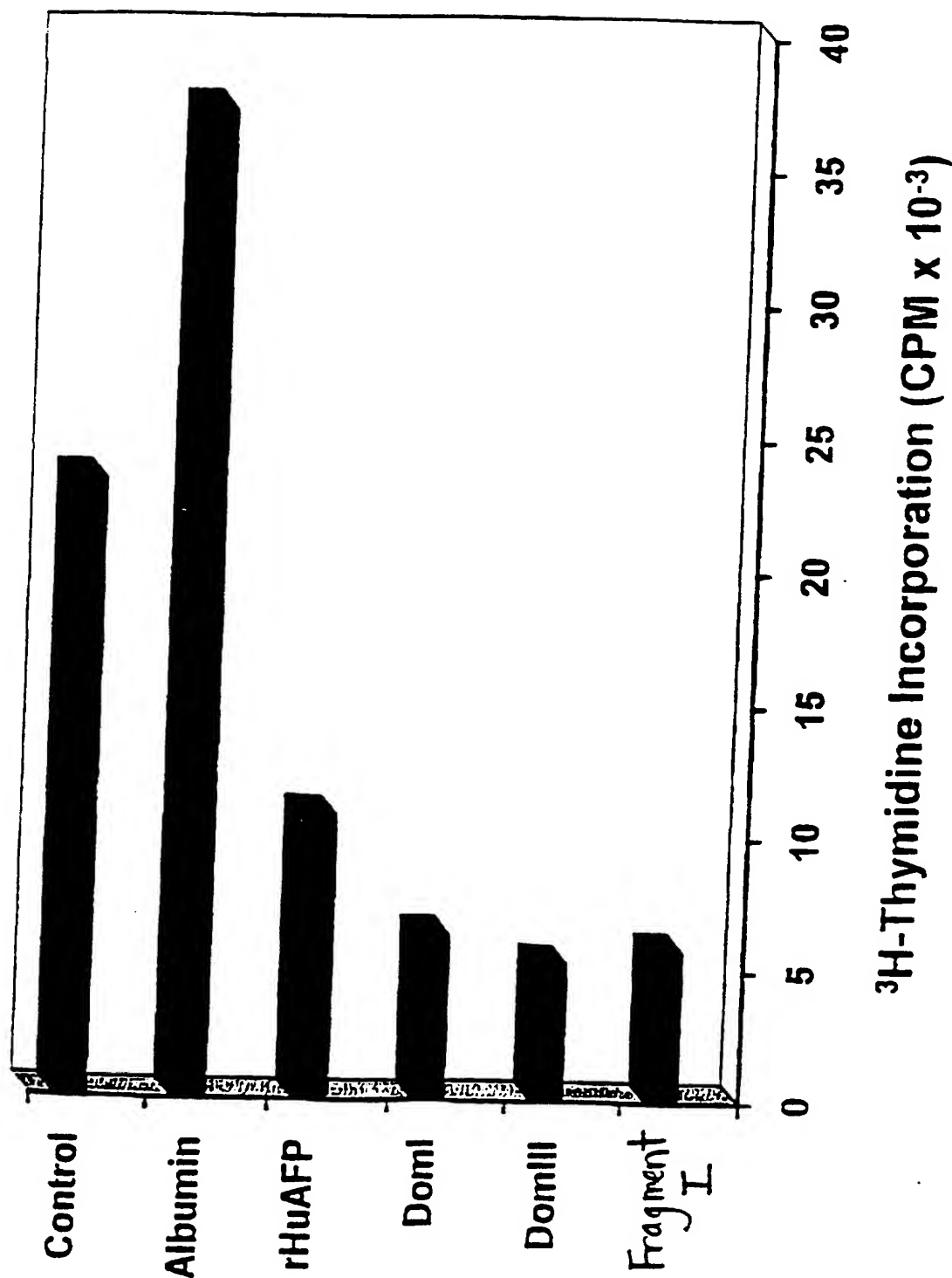
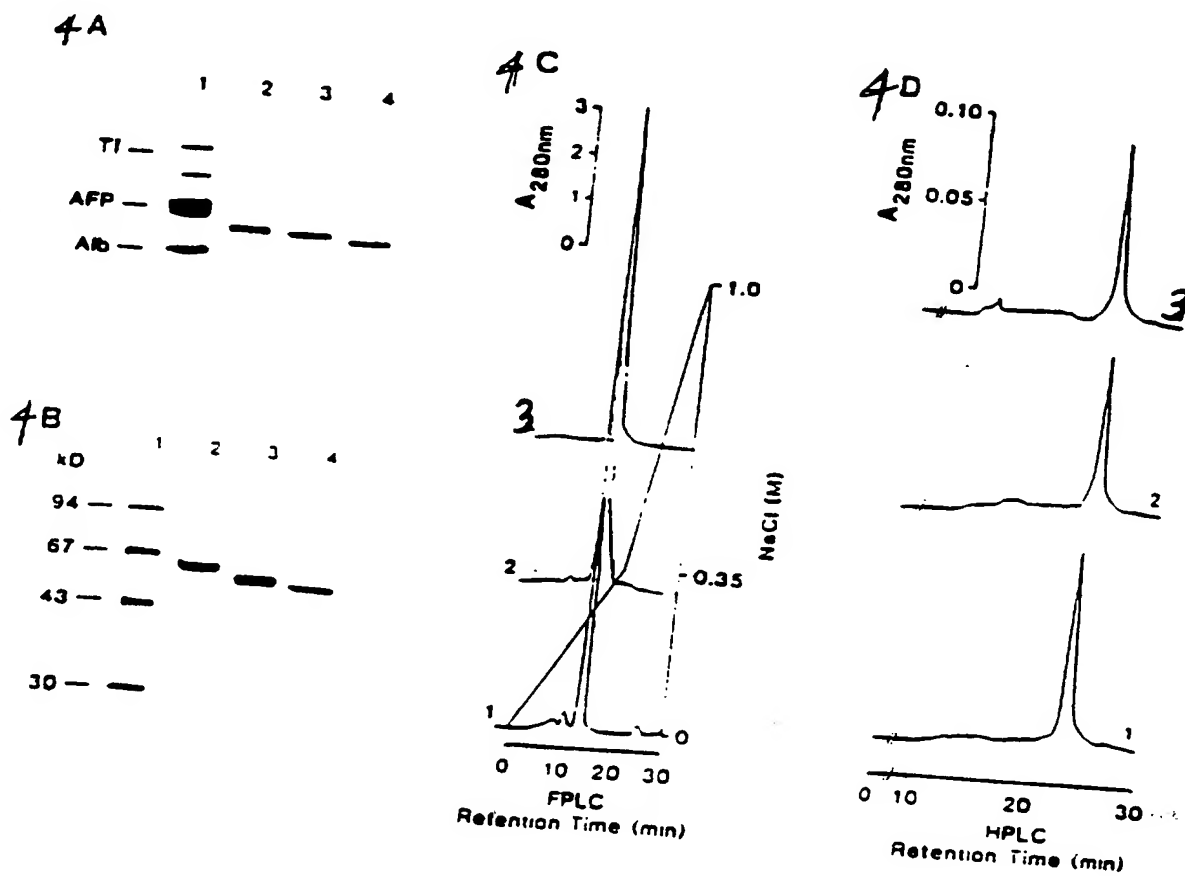


Figure 3

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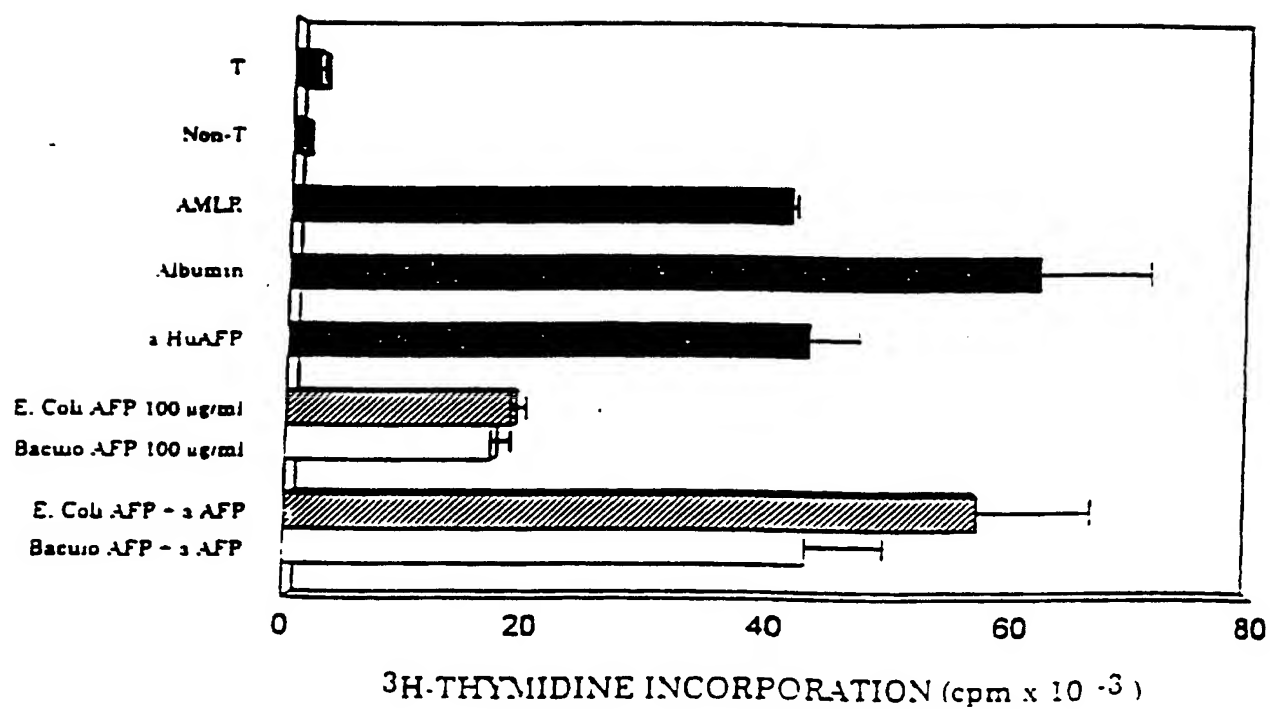
Figure 4





5/10

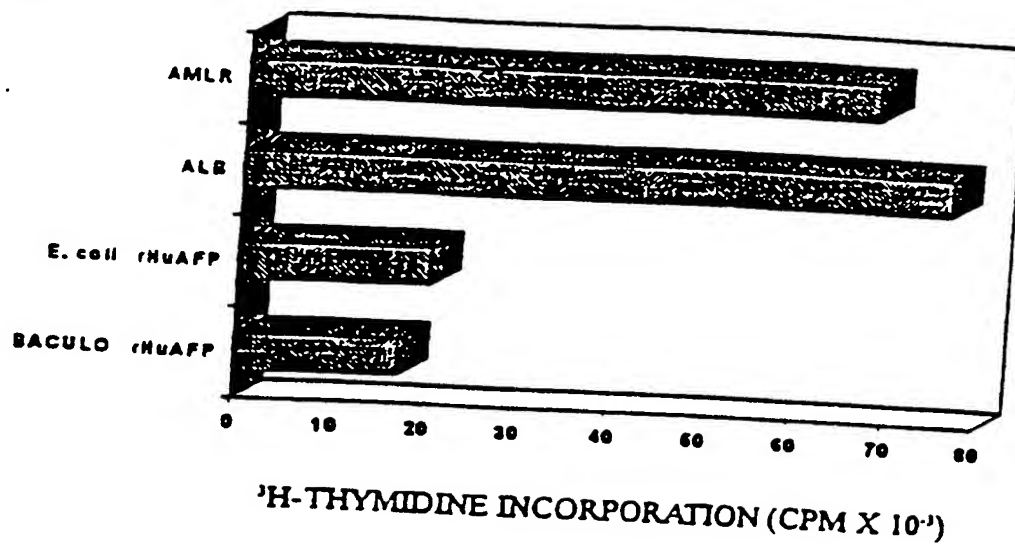
Figure 5



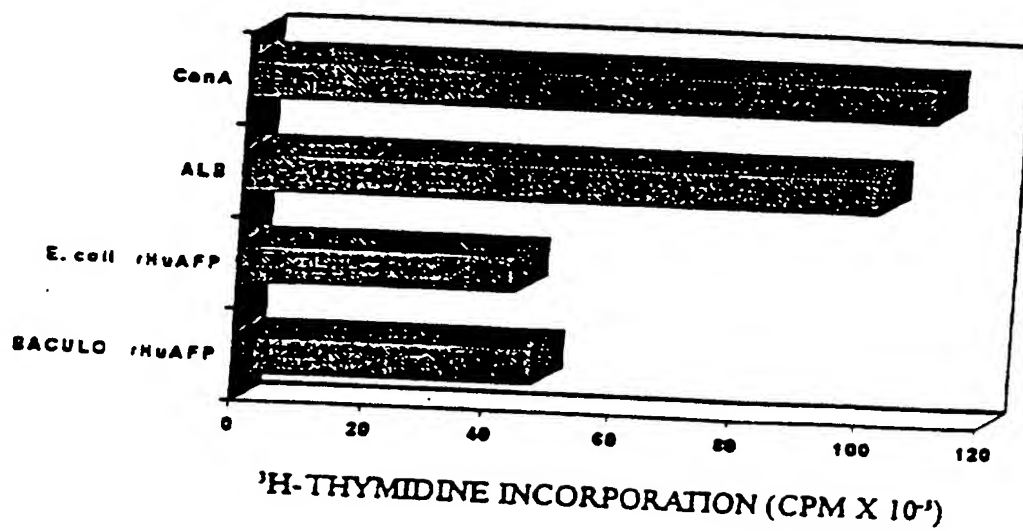
6/10

Figure 6

6A

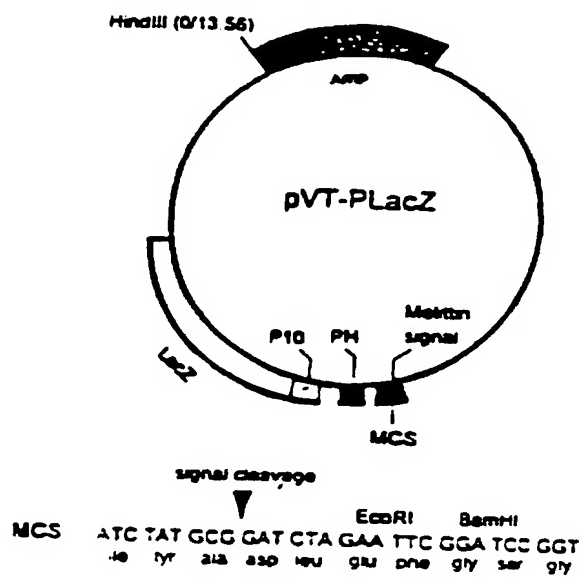


6B



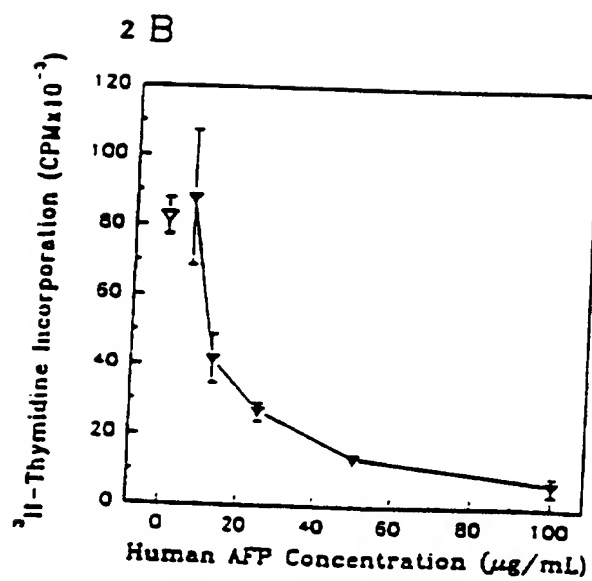
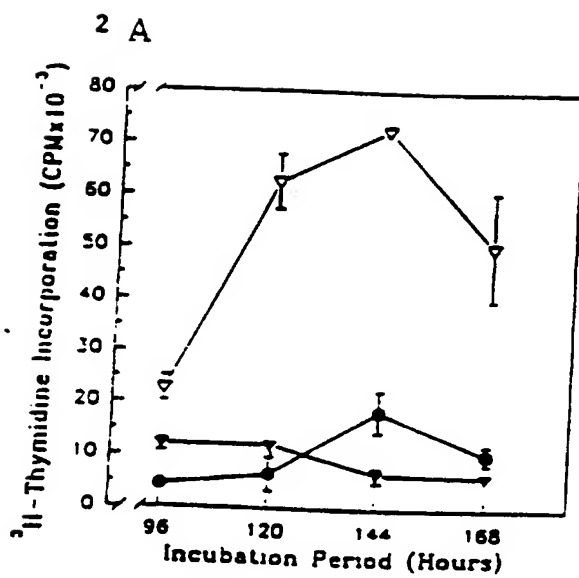
7/10

Figure 7



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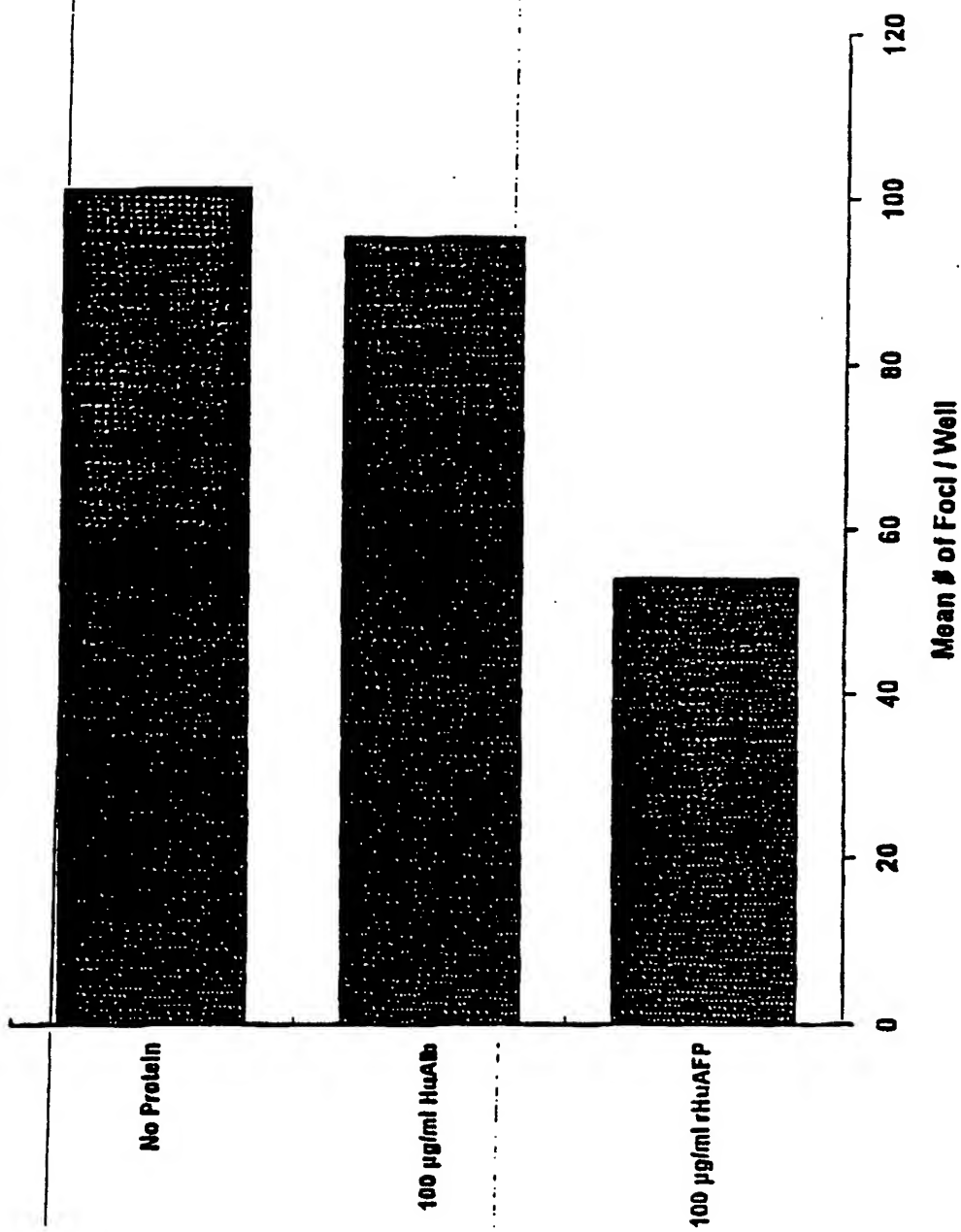
Figure 8



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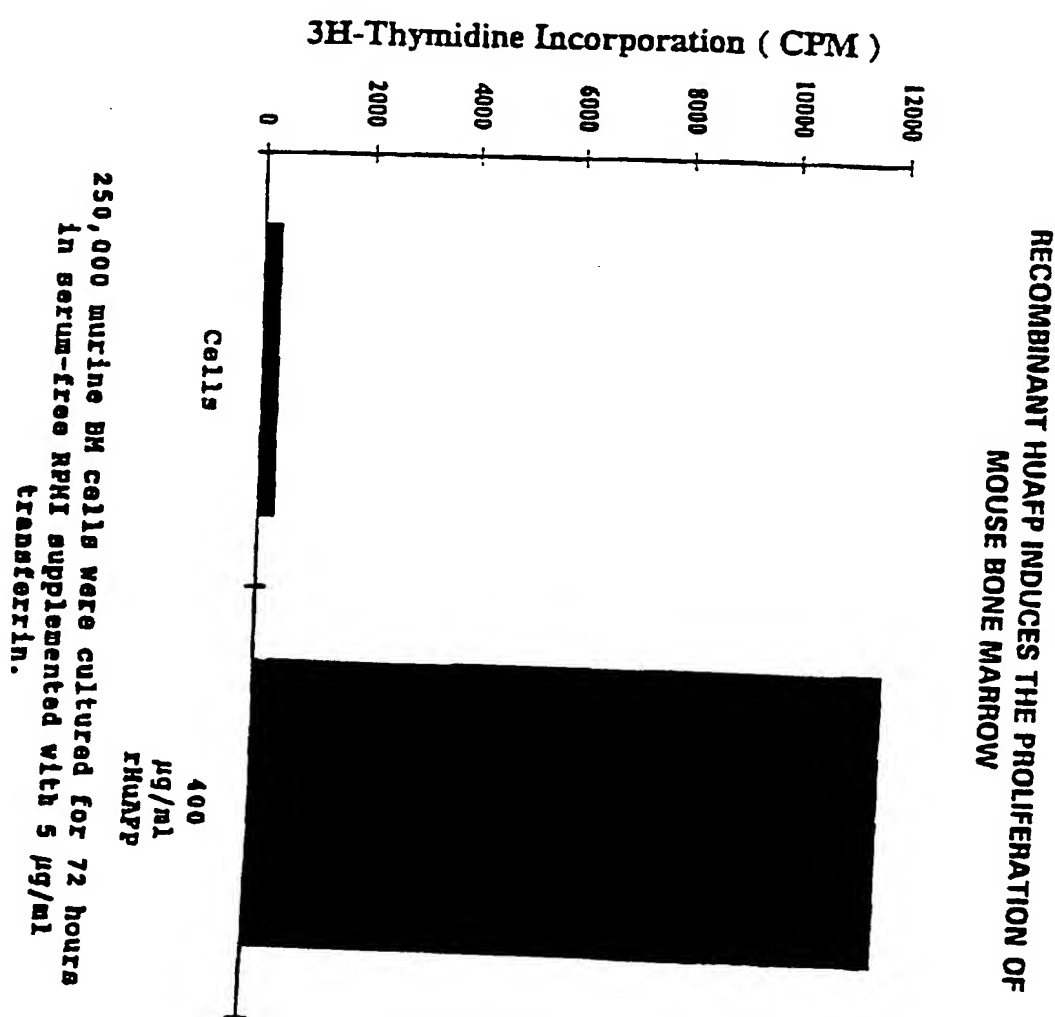
Figure 9

**EFFECT OF RECOMBINANT HUMAN AFP ON ESTROGEN-STIMULATED POST-CONFLUENT  
GROWTH OF MCF-7 HUMAN BREAST CANCER CELLS**



10/10

Figure 10



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/00996**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 38/17; C07K 14/00; C12P 21/02

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.6, 70.1, 70.3, 71.2; 514/2, 825, 866, 885, 903; 530/350, 380; 930/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 94/10199 A1 (MCGILL UNIVERSITY) 11 May 1994, see entire document.	1-10, 12-15 ----- 16-35
Y	US 5,342,625 A (HAUER ET AL) 30 August 1994, column 1, line 52 to column 2, line 10.	30, 32-34
Y	US 5,011,844 A (T. FEHR) 30 April 1991, column 8, lines 17-30.	30, 32, 33, 35
Y	BENJAMIN et al., "Induction of tolerance by monoclonal antibody therapy". Letters to Nature. 03 April 1985, Vol. 320, pages 449-451, especially pages 449 and 451.	31

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A document defining the general state of the art which is not considered to be part of particular relevance	*X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E earlier document published on or after the international filing date	*Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z document member of the same patent family
*O document referring to an oral disclosure, use, exhibition or other means	
*P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 10 MAY 1996	Date of mailing of the international search report 20 MAY 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer STEPHEN GUCKER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1992)\*

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/00996

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MURGITA et al., "Effects of human alpha-foetoprotein on human B and T lymphocyte proliferation in vitro". Clin. Exp. Immunol. 1978, Vol. 33, pages 347-356, especially page 354.	16-35
Y	HOSKIN et al., "In Vitro Activation of Bone Marrow-Derived T- and Non-T-Cell Subsets by alpha-fetoprotein". Cellular Immunology, 1985, Vol. 96, pages 163-174, especially page 173.	16-35



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/00996

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/69.1, 69.6, 70.1, 70.3, 71.2; 514/2, 825, 866, 885, 903; 530/350, 380; 930/10

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, SCISEARCH, EMBASE, BIOSIS, CAPLUS, BIOTECHDS, DISSABS, CONFSCI, LIFESCI  
search terms: fetoprotein, proliferat####, autoimmune, sclerosis, arthritis, myasthenia, diabetes, lupus, cyclosporine,  
FK-506

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-10, 12-35, drawn to alpha-fetoprotein, therapeutic compositions containing alpha-fetoprotein, method of making alpha-fetoprotein recombinantly, and a method to treat autoimmune diseases by using alpha-fetoprotein.

Group II, claims 36-53, drawn to a method of treating neoplasms by using alpha-fetoprotein.

Group III, claims 54-57, drawn to a hybrid cytotoxin wherein alpha-fetoprotein is part of a targeted fusion protein.

Group IV, claims 58-86, drawn to methods of imaging, detecting, and diagnosing neoplasms by using detectably-labelled alpha-fetoprotein and the detectably-labelled alpha-fetoprotein itself.

Group V, claims 87-97, drawn to a method of cell culture using alpha-fetoprotein to stimulate proliferation and the cell culture medium itself.

Group VI, claims 98-110, drawn to methods of treating bone marrow cell disorders by using alpha-fetoprotein.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I-VI do not share a special technical feature because alpha-fetoprotein, which is the technical feature and inventive link shared by Groups I-VI, does not define a contribution over the prior art as defined in PCT Rule 13.2.

Group I is drawn to the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein.

Group II is drawn to the special technical feature of a method of treating neoplasms by using alpha-fetoprotein, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

Group III is drawn to the special technical feature of a hybrid cytotoxin wherein alpha-fetoprotein is part of a targeted fusion protein, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

Group IV is drawn to the special technical feature of methods of imaging, detecting, and diagnosing neoplasms by using detectably-labelled alpha-fetoprotein and the detectably-labelled alpha-fetoprotein itself, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

Group V is drawn to the special technical feature of a method of cell culture using alpha-fetoprotein to stimulate proliferation and the cell culture medium itself, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

Group VI is drawn to the special technical feature of methods of treating bone marrow cell disorders by using alpha-fetoprotein, and does not share the special technical feature of a method of treating autoimmune disease by using alpha-fetoprotein of Group I.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US96/00996

Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.